Ecotoxicology (2009) 18:440–446
DOI 10.1007/s10646-009-0298-1

Toxicity of soybean rust fungicides to freshwater algae and *Daphnia magna*

Hugo G. Ochoa-Acuña · Walter Bialkowski · Gowri Yale · Leighanne Hahn

Accepted: 12 January 2009 / Published online: 31 January 2009

Abstract Soybeans are intensively grown over large swaths of land in the Midwestern US. Introduction of the pathogenic fungus responsible for Soybean Rust (*Phakopsora pachyrhizi*) will likely result in a significant increase in the environmental load of strobilurin and conazole fungicides. We determined the toxicity of six such fungicides to the unicellular algae *Pseudokirchneriella subcapitata* and the aquatic invertebrate, *Daphnia magna*. We found that levels of concern of some fungicides were lower than annual average runoff concentrations predicted for Indiana. Our results suggest that pyraclostrobin and propiconazole, and to a lesser extent tebuconazole, may cause impacts to algae and daphnids in areas where soybeans are intensively grown. More studies are needed to describe the ecological effects of sublethal exposures to these fungicides, as well as monitoring environmental concentrations in watersheds where these fungicides are applied to soybeans.

Keywords Conazole · Strobilurin · Soybean rust · *Pseudokirchneriella* · *Daphnia magna*

Introduction

The recent introduction of soybean rust into the US has been a cause of concern because this fungus is a significant pathogen of soybeans, associated with yield losses ranging from 10 to 80% (Nunkumar et al. 2006). In response to this introduction, the US Environmental Protection Agency (USEPA) granted Sect. 18 Emergency Quarantine Exemption to several fungicides previously unavailable for use on soybeans. These pesticides belong to two groups: strobilurin and azole fungicides.

Strobilurin fungicides act by inhibiting mitochondrial respiration by binding to the Q_o site of cytochrome *b*, an important part of a mitochondrial membrane complex involved in energy transfer in eukaryotes (Bartlett et al. 2002). The transfer of electrons between parts of this complex is disrupted when the fungicides bind and prevent the formation of adenosine triphosphate (Bartlett et al. 2002). To our knowledge, there is no information available in the peer-reviewed literature on the toxicity of strobilurin fungicides to non-target species. However, the data made available for registration show that toxic effects accrue within a narrow range of concentrations, probably due to the toxic mechanism involved (Bartlett et al. 2002).

The mode of action of azole fungicides is through binding to the heme protein of fungal CYP51 C-14, inhibiting demethylation in ergosterol biosynthesis (Venkatakrishnan et al. 2000). This mode of action may result in potential adverse effects of conazoles on CYP-mediated processes in non-target species. In addition, a recent study found that many azole fungicides showed identical metabolite profiles among rat and trout chromosomes that also matched the profile observed using purified human CYP 3A4 (Mazur and Kenneke 2008). These results suggest that adverse effects mediated through this mode of action may be of significance in non-target species.

Baseline toxicity data are not available from peer-reviewed studies for strobilurin or conazole fungicides that have been granted Sect. 18 Emergency Exemption by the USEPA for use in soybeans. Application of these

H. G. Ochoa-Acuña (✉) · W. Bialkowski · G. Yale
Department of Comparative Pathobiology, Purdue University, 725 Harrison St., West Lafayette, IN 47907, USA
e-mail: hochoaac@purdue.edu

L. Hahn
Office of the Indiana State Chemist, Purdue University, 175 S. University St., West Lafayette, IN 47907, USA

fungicides to soybeans may result in significant environmental releases. A recent study conducted by the USDA (Livingston et al. 2004) estimated that conditions in the Midwest would be favorable for the development of soybean rust during most years. In addition, the management recommendation for this disease is to use fungicides at their maximum allowable rate to prevent development of resistant fungal strains.

In states such as Illinois, Iowa, and Indiana, more than 24% of the total land area is planted each year with soybeans (United States Department of Agriculture (USDA) 2008). Because of the large acreage planted with this crop, control of soybean rust will likely result in large amounts of fungicides entering aquatic environments through runoff. A recent study predicted the potential concentration in field runoff of these fungicides for Indiana based on their recommended application patterns (Deb 2007). However, interpretation of these results was hindered by the lack of effects data on ecologically relevant endpoints and species. The objective of the present study was to provide toxicity information of these fungicides to algae and *Daphnia magna* and predict the potential ecological impact of using these fungicides to combat soybean rust.

Materials and methods

Test organisms

The green alga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*), was obtained from Carolina Biological Supplies (Burlington, NC). Algae were cultured in standard medium, as proposed by the United States Environmental Protection Agency (USEPA) (2002). Prior to use, the medium was sterilized by ultrafiltration using 0.45 μm mesh size filters. Culturing was conducted at 25 ± 1°C under continuous cool-white fluorescent illumination with a light intensity of 4,000 lux.

Daphnia magna Straus, originally obtained from Aquatic Biosystems Inc. (Fort Collins, CO, USA), were cultured in 3-l plastic aquaria containing a modified high-hardness COMBO medium (Baer and Goulden 1998). Animals were subject to a 16:8 light:dark cycle using fluorescent, cool-white light and temperature was maintained at 22 ± 1°C.

Test conditions

All pesticide solutions were prepared immediately prior to the initiation of testing and each water exchange. Technical grade propiconazole was procured from Sigma Aldrich (St Louis, MO, USA). Azoxystrobin, trifloxystrobin, pyraclostrobin, tebuconazole, and tetraconazole were procured from AccuStandard, Inc. (New Haven, CT, USA). No solvent was used as stock and exposure concentrations were well below the solubility of each fungicide in water. Reported concentrations are nominal values.

Algal-growth inhibition tests were conducted using four replicates per exposure level. We used the microplate method for testing toxicity of fungicides to algae (Blaise and Vasseur 2005). Tests were conducted in 96-well plates, with each well containing 200 μl of test solution. Immediately before the start of the inhibition test, each well was inoculated with 20 μl of an algal suspension containing $\sim 1.2 \times 10^5$ cells/ml. Algal concentrations were then determined using a Bio-Tek Synergy HT Multidetection Microplate reader using both absorbance (at 490 nm), and fluorescence (excitation at 485 nm and detection at 670 nm) measurements. Calibration curves were checked for accuracy during each plate reading by determining absorbency and fluorescence algal suspensions whose concentration was previously determined by direct counting using a hemocytometer.

One-day old *D. magna* used for testing were isolated from adults 16 to 21 days old (United States Environmental Protection Agency (USEPA) 2002). Prior to testing, D. magna neonates were held in a 1-l aquarium for 2 h and fed Instant Algae Nanno 3600^{TM} (Reed Mariculture, San Jose, CA, USA) and YCT (United States Environmental Protection Agency (USEPA) 2002). Five animals were then transferred to glass beakers containing 50 ml test medium. Four replicates were run for each treatment concentration. Animals were transferred into beakers with fresh solution and fed daily. Mortality was assessed after 24, 48, 72, and 96 h of exposure.

At least one preliminary test was performed for all compounds tested. If 100% inhibition or mortality was observed in more than one of the range-finding test concentrations, then the range of concentrations was narrowed and the highest doses eliminated from further testing. We report the median and tenth percentile inhibition (algae) and lethal (*D. magna*) concentrations (IC_{50}/LC_{50} and IC_{10}/LC_{10}, respectively). We used PROC PROBIT to estimate IC_{50}/LC_{50} and IC_{10}/LC_{10} and their confidence intervals (SAS Institute, Inc. 2008).

Results

Percent inhibition curves of *P. subcapitata* in relation to exposure concentration to the fungicides under study are shown in Fig. 1. The median and 10th percentile inhibition concentrations for each fungicide tested are presented in Table 1. The fungicide with the lowest IC_{10} and IC_{50} was trifloxystrobin, followed by propiconazole and azoxystrobin. The least toxic fungicides to algae were tebuconazole and tetraconazole. There was also a large variation in the factor separating the IC_{10} and the IC_{50}. The smaller factors corresponded to tebucanozole (~3) and azoxystrobin (~7); whereas the largest factor between IC_{50} and IC_{10} corresponded to propiconazole and trifloxystrobin (~55 and ~21, respectively).

The percent mortality of *D. magna* exposed to the fungicides at different exposure times is shown in Fig. 2. The median and 10th percentile mortality rates for each exposure period and fungicide are shown in Table 2. In the case of tebuconazole, only data from 48 h exposures were available. As shown in Table 2, the most toxic fungicide to *D. magna* at 48 h was pyraclostrobin. The two least toxic fungicides were propiconazole and tetraconazole.

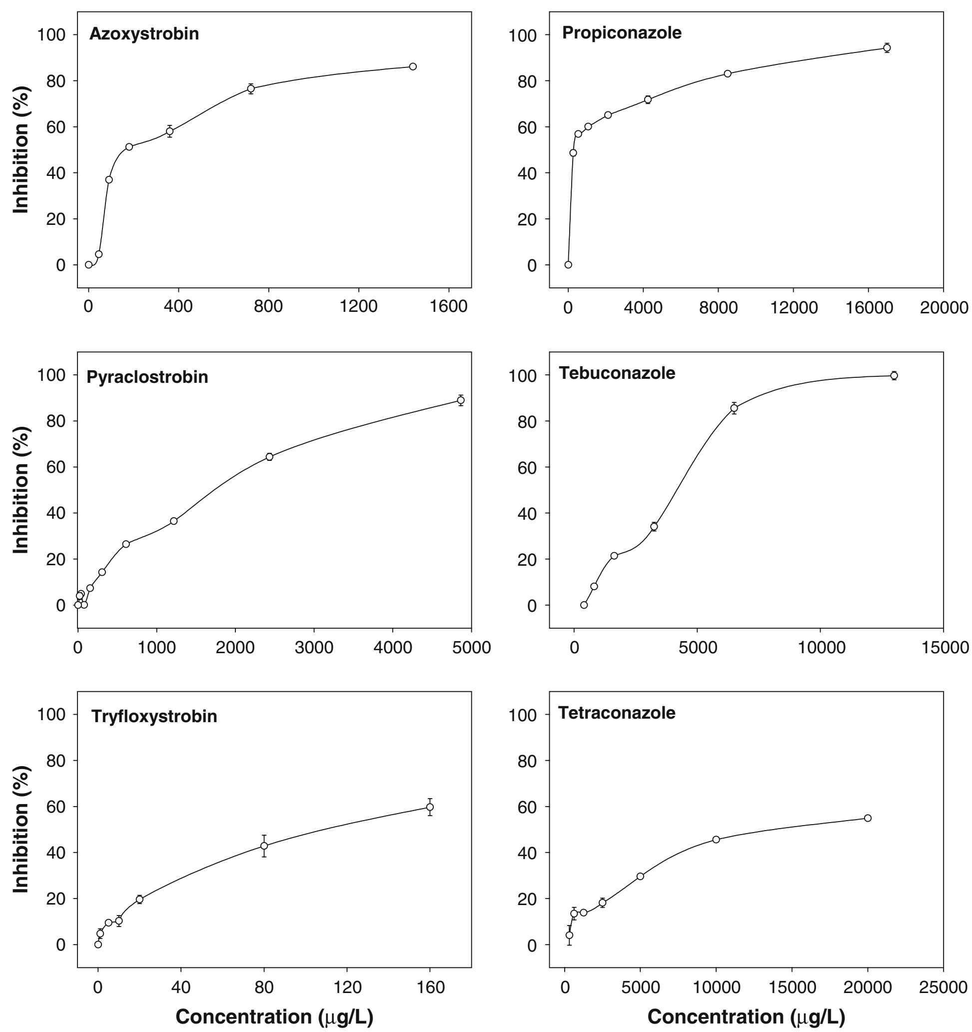
In addition to dose-dependent responses that were observed for all four fungicides tested, a time-dependent relationship was also observed. Although the LC_{50} for azoxystrobin, tetraconazole, and trifloxystrobin did not change significantly from 24 to 96 h exposures, the toxicity of propiconazole and pyraclostrobin were highly dependent on the length of exposure. For example, propiconazole was slightly to moderately toxic after 48 h (LC_{50} = 9,000 μg/l), but highly toxic (LC_{50} = 180 μg/l) after 96 h of exposure.

Table 1 Summary of median (IC_{50}) and tenth percentile (IC_{10}) inhibition concentrations (μg/l) of azoxystrobin, propiconazole, pyraclostrobin, tebuconazole, tetraconazole, and trifloxystrobin to *Pseudokirchneriella subcapitata* after 72 h

Pesticide/Endpoint	Endpoint	
	IC_{50}	IC_{10}
Azoxystrobin	230 (190–270)	32 (21–44)
Propiconazole	390 (220–590)	6.8 (1.1–20)
Pyraclostrobin	1,400 (1,200–1,600)	250 (180–320)
Tebuconazole	3,200 (2,900–3,600)	1,200 (1,000–1,400)
Tetraconazole	15,000 (11,000–23,000)	950 (460–1,500)
Trifloxystrobin	120 (80–190)	5.7 (3.4–8.3)

Toxicity endpoints were calculated based on nominal concentrations. Values in parenthesis correspond to the 95% confidence interval of each estimate

Fig. 1 Growth inhibition (%) of *Pseudokirchneriella subcapitata* after 72 h in response to azoxystrobin, propiconazole, pyraclostrobin, tebuconazole, tetraconazole, and trifloxystrobin



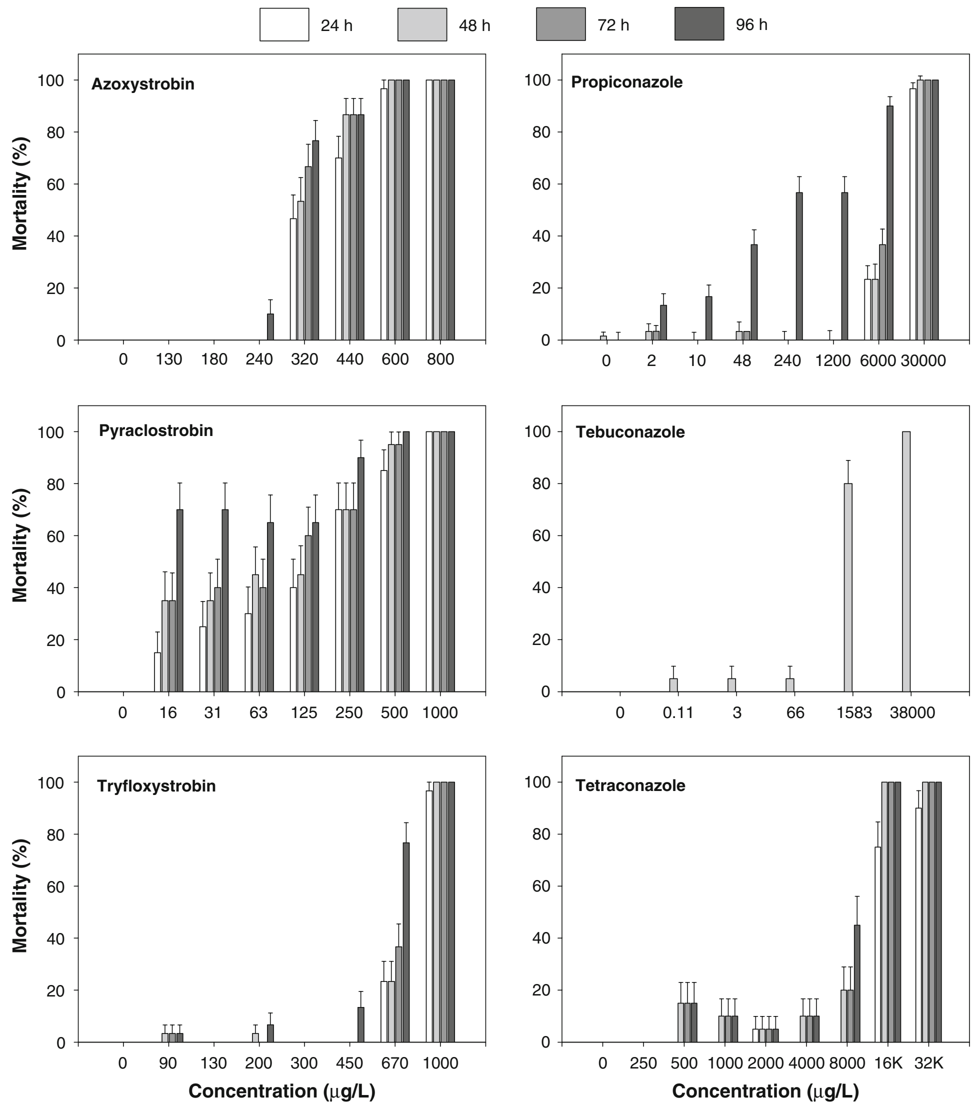


Fig. 2 Mortality (%) of *Daphnia magna* in response to azoxystrobin, propiconazole, pyraclostrobin, tebuconazole, tetraconazole, and trifloxystrobin exposures of 24, 48, 72, and 96 h

Discussion

We found that toxicity varied among the six fungicides studied. Trifloxystrobin demonstrated a higher level of toxicity to algae when considering either the IC_{50} or IC_{10} as indicators of threshold toxicity. In the case of *D. magna*, pyraclostrobin was the most toxic fungicide after 96 h of exposure with the lowest LC_{50} and LC_{10}. The IC_{50} of algae exposed to trifloxystrobin was similar to that of propiconazole, but *D. magna* were significantly less sensitive to this fungicide than to propiconazole. For some fungicides, there was a marked reduction in the calculated LC_{50} and LC_{10} when considering longer exposure periods. This suggests that toxicity evaluations may vary considerably for some fungicides (e.g., propiconazole) based on the length of the exposure period.

We decided not to report lowest and no effect concentrations (NOEC and LOEC, respectively) because these are prone to bias based on the selection of concentrations to be tested and because they do not take into account the entire concentration–effect relationship. Given that it is customary to allow a 10% inhibition or mortality to occur in the control group, the IC_{10}/LC_{10} and its lower 95% confidence interval have been proposed to represent endpoints similar to the LOEC and NOEC, respectively (United States Environmental Protection Agency (USEPA) 1995).

There is considerable information (especially for conazole fungicides) on toxic effects to vertebrates. Aside from the limited information available from product labels/MSDS sheets (reviewed by Spradley et al. 2005), very little is known regarding the toxicity of these fungicides to freshwater invertebrates and algae. A study conducted on the Pacific white shrimp (*Litopenaeus vannamei*) exposed to propiconazole found that the 72-h LC_{50} was 1,167 (1,101–1,386) µg/l (Betancourt-Lozano et al. 2006). A recent study found that pyraclostrobin was highly toxic to

Table 2 Summary of median (LC_{50}) and tenth percentile (LC_{10}) lethal concentrations (μg/l) of azoxystrobin, propiconazole, pyraclostrobin, tebuconazole, tetraconazole, and trifloxystrobin to *Daphnia magna* exposed for 24, 48, 72, and 96 h

Pesticide/endpoint	Exposure duration			
	24 h	48 h	72 h	96 h
Azoxystrobin				
LC_{50}	370 (340–390)	340 (320–360)	330 (300–350)	310 (280–330)
LC_{10}	260 (230–290)	260 (230–290)	250 (220–280)	230 (200–250)
Propiconazole				
LC_{50}	9,500 (7,200–13,000)	9,000 (5,100–19,000)	6,800 (4,000–13,000)	180 (92–350)
LC_{10}	4,300 (2,500–5,800)	630 (260–1,200)	530 (220–980)	2.7 (0.6–7.4)
Pyraclostrobin				
LC_{50}	120 (95–140)	68 (40–93)	79 (52–100)	14 (4.5–26)
LC_{10}	17 (8.9–27)	2.6 (0.33–7.6)	4.2 (0.85–10)	0.71 (0.06–2.6)
Tebuconazole				
LC_{50}		750 (320–2,000)		
LC_{10}		6.2 (1.4–18)		
Tetraconazole				
LC_{50}	14,000 (11,000 –18,000)	7,200 (5,200–10,000)	7,200 (5,200–10,000)	5,900 (4,400–8,400)
LC_{10}	6,000 (3,900–7,900)	1,500 (830–2,200)	1,500 (830–2,200)	1,300 (770–1,900)
Trifloxystrobin				
LC_{50}	750 (700–810)	740 (640–890)	690 (610–810)	530 (470–610)
LC_{10}	610 (530–660)	380 (300–440)	360 (290–420)	290 (230–330)

Toxicity endpoints were calculated based on nominal concentrations. Values in parenthesis correspond to the 95% confidence interval of each estimate

glochidia and juveniles of freshwater mussels (*Unionidae*), with LC_{50} s ranging from 30 to 80 μg/l (Bringolf et al. 2007). The only study available in the peer-reviewed literature regarding effects of these fungicides to algae showed that exposure to 83 μg/l propiconazole resulted in a 13% growth inhibition of *Pseudokirchneriella subcapitata* (Peterson et al. 1994). These limited data do not differ significantly with the data collected in this study.

The introduction of soybean rust in the US is likely to result in a significant increase in the amount of fungicides released into the environment. In the Midwest, a large proportion of land is devoted to growing soybeans each year. In 2008, 24% of Indiana was planted with this crop (United States Department of Agriculture (USDA) 2008). A recent study using National Agricultural Pesticide Risk Analysis (NAPRA) predicted concentrations in runoff of fungicides used to combat soybean rust. Based on application rates, physical/chemical properties of each fungicide, and Indiana's weather and soil characteristics, the model predicted the concentration of pesticides in runoff at edge of field (Deb 2007) for 100 years modeled. This modeling exercise predicted that edge of field annual average runoff concentrations for azoxystrobin, trifloxystrobin, and tetraconazole would be significantly lower than levels of concern reported here. However, this study found that, during 5 out of 100 years, the annual average concentration of propiconazole in runoff would vary between 13 and 42 μg/l, depending of the region of Indiana considered. These concentrations are lower than the IC_{50} and LC_{50} calculated by us for algae and *D. magna*, respectively. However, they significantly exceed the IC_{10} and LC_{10}. Deb (2007) also calculated very similar values for tebuconazole (14–46 μg/l), which exceed the 48-h LC_{10} calculated by us. In the case of pyraclostrobin, concentration ranges would be 1.5–14 μg/l, which approach the LC_{50} for *D. magna*, but are lower than levels of concern for algae. Therefore, algae inhibition and some daphnid mortality is predicted to occur at least in some localized areas during some years due to use of propiconazole and tebuconazole. Pyraclostrobin, however, although not expected to cause adverse effects to algal populations under the assumptions used by Deb (2007), appears to have the potential for reaching acutely toxic levels to daphnids in some areas.

Several sources of uncertainty need to be considered when drawing conclusions from comparing our results with the projections made by Deb (2007). A major source of uncertainty is that our estimated endpoints correspond to short-term exposures, whereas the runoff concentrations calculated by Deb (2007) correspond to annual averages.

On the other hand, these concentrations are applicable to edge of fields, before any dilution with un-impacted water has occurred. If pyraclostrobin were to be extensively used in soybeans, then our results and those of Deb (2007) would suggest that concentrations of concern may be reached in some areas, given the significant proportion of land planted with soybeans every year in many Midwestern watersheds. On the other hand, our laboratory exposures where conducted using standard media under controlled conditions, with tests solutions made immediately before use. Environmental exposures may differ markedly from laboratory exposures and degradation of products may occur before adverse effects are elicited. For example, pyraclostrobin is known to photodegrade quite rapidly in aqueous solution and therefore it may quickly disappear from runoff.

Another source of uncertainty of our results is that endpoints were calculated using nominal, instead of measured, concentrations. We attempted to minimize this potential source of uncertainty by preparing fresh solutions before test commencement and each water change, and use of amber glass wrapped in aluminum foil. The limited data available suggest that propiconazole, tetraconazole, and tebuconazole are very stable in water, with half lives when exposed to sunlight ranging from 60 to >250 days (Australian Pesticides and Veterinary Medicines Authority (APVMA) 2005; European Food Safety Authority (EFSA) 2003). Strobilurins, on the other hand, seem to be more photolabile, with half lives of 9–14 days for azoxystrobin, and 1–2 days for pyraclostrobin and tryfloxystrobin (Australian Pesticides and Veterinary Medicines Authority (APVMA) 2000; European Food Safety Authority (EFSA) 1998, 2004).

Our results are directly applicable to two critical functional components of freshwater ecosystems: primary producers and planktonic filter feeders. Given their key roles, effects on these organisms may result in significant ecosystem impairment. Furthermore, we showed that significant mortality and algal growth inhibition may be elicited by concentrations expected to prevail during significant periods. This is significant because other ecologically relevant effects associated to chronic exposures (e.g., effects on growth and reproduction) are likely to occur at lower exposure concentrations. Our results for *D. magna* may also signify potential effects to other aquatic organisms. *D. magna* has been shown to be moderately sensitive to organic chemicals with several invertebrate taxa (Orders Amphipoda and Plecoptera, as well as other cladocerans) being more sensitive (Wogram and Liess 2001). In addition, our data for *D. magna* compare well with those for the fatmuket mussel (*Lampsilis siliquoidea*) obtained by Bringolf et al. (2007). This is significant because several Midwestern watersheds still harbor mussel species listed as endangered. Endangered mussels may not only be impacted directly by some of these fungicides, but also indirectly through a decrease in filterable unicellular algae concentrations.

In conclusion, there is potential for ecological impacts from the use of some soybean rust fungicides in watersheds where a significant proportion of the land is planted with soybeans and treated with these pesticides. Further studies on the potential for these fungicides having population impacts through chronic effects on reproduction and growth should be conducted. In addition, monitoring of fungicide concentrations in surface waters where these products are applied would allow determining whether modeled concentrations are adequate for predicting environmental exposures.

References

Australian Pesticides and Veterinary Medicines Authority (APVMA) (2000) Evaluation of the new active trifloxistrobin in the product Flint Fungicide. http://www.apvma.gov.au/publications/downloads/prstri.pdf. Accessed 5 January 2009

Australian Pesticides and Veterinary Medicines Authority (APVMA) (2005) Evaluation of the new active tetraconazole in the product prstetra.pdf. Accessed 5 January 2009

Baer KN, Goulden CE (1998) Evaluation of a high-hardness COMBO medium and frozen algae for *Daphnia magna*. Ecotoxicol Environ Saf 39:201–206. doi:10.1006/eesa.1997.1627

Bartlett DW, Clough JM, Godwin JR et al (2002) Review: the strobilurin fungicides. Pest Manag Sci 58:649–662. doi:10.1002/ps.520

Betancourt-Lozano M, Baird DJ, Sangha RS et al (2006) Induction of morphological deformities and moulting alterations in *Litopenaeus vannamei* (Boone) juveniles exposed to the triazole-derivative fungicide Tilt. Arch Environ Contam Toxicol 51:69–78. doi:10.1007/s00244-005-0149-x

Blaise C, Vasseur P (2005) Algal microplate toxicity test. In: Blaise C, Férard JF (eds) Small-scale freshwater toxicity investigations, volume 1—toxicity test methods. Springer, Dordrecht, pp 137–180

Bringolf RB, Cope WG, Eads CB et al (2007) Acute and chronic toxicity of technical-grade pesticides to glochidia and juveniles of freshwater mussels (*Unionidae*). Environ Toxicol Chem 26:2086–2093. doi:10.1897/06-522R.1

Deb D (2007) Estimating environmental exposure of emerging agricultural contaminants using spatial data analysis and geographic information system. Doctoral Dissertation, Purdue University. 148 p

European Food Safety Authority (EFSA) (1998) Review report for the active substance azoxystrobin. http://ec.europa.eu/food/plant/protection/evaluation/newactive/list2-01_en.pdf. Accessed 5 January 2009

European Food Safety Authority (EFSA) (2003) Review report for the active substance propiconazole. http://ec.europa.eu/food/plant/protection/evaluation/existactive/list1-51_en.pdf. Accessed 5 January 2009

European Food Safety Authority (EFSA) (2004) Review report for the active substance pyraclostrobin. http://ec.europa.eu/food/plant/protection/evaluation/newactive/pyraclostrobin.pdf. Accessed 5 January 2009

Livingston M, Johansson R, Daberkow S et al. (2004) Economic and policy implications of wind-borne entry of Asian soybean rust into the United States. United States Department of Agriculture. Outlook Report N0 OCS04D02. http://www.ers.usda.gov/Publications/OCS/Apr04/OCS04D02/. Accessed 5 January 2009

Mazur CS, Kenneke JF (2008) Cross-species comparison of conazole fungicide metabolites using rat and rainbow trout (*Onchorhychus mykiss*) hepatic microsomes and purified human CYP 3A4. Environ Sci Technol 42:947–954. doi:10.1021/es072049b

Nunkumar A, Caldwell PM, Pretorius ZA (2006) Studies on soybean rust (*Phakopsora pachyrhizi*). Phytopathology 96:1164

Peterson HG, Boutin C, Martin PA et al (1994) Aquatic Phytotoxicity of 23 pesticides applied at expected environmental concentrations. Aquat Toxicol 28:275–292. doi:10.1016/0166-445X(94)90038-8

SAS Institute, Inc. (2008) SAS/STAT 9.2 User's guide. SAS Institute, Inc., Cary

Spradley PCR, Goodwin AE, Selden GL (2005) Asian soybean rust fungicides and aquatic organisms. FSA7532–1 M-10_05RV. University of Arkansas Division of Agriculture, Cooperative Extension Service, Little Rock

United States Department of Agriculture (USDA) (2008). Acreage report June 2008. http://usda.mannlib.cornell.edu/usda/current/Acre/Acre-06-30-2008.pdf. Accessed 5 January 2009

United States Environmental Protection Agency (USEPA) (1995) The use of the benchmark dose approach in health risk assessment. ORD, Washington (EPA/630/R-94/007)

United States Environmental Protection Agency (USEPA) (2002) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th edn. United States Environmental Protection Agency, Washington

Venkatakrishnan K, von Moltke LL, Greenblatt DJ (2000) Effects of the antifungal agents on oxidative drug metabolism: clinical relevance. Clin Pharmacokinet 38:111–180. doi:10.2165/00003088-200038020-00002

Wogram J, Liess M (2001) Rank ordering of macroinvertebrate species sensitivity to toxic compounds by comparison with that of *Daphnia magna*. Bull Environ Contam Toxicol 67:360–367