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Cytotoxicity of Technical Grade versus Formulations of Atrazine and Acetochlor Using Mammalian Cells

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Since the mid 20th century, selective herbicides have been an increasingly important part of modern agriculture. The increased usage can be attributed to reduced labor and time, which translates to lower economic input. The use of herbicides also has environmental benefits. No-till agriculture (to which herbicides play a significant role) results in high productivity with minimum soil erosion (Vetsch and Randall 2002). However the use of herbicides does have potential problems. No-till agriculture results in an increase of herbicide contamination (Malone et al. 2003). As herbicide use increased, contamination of watersheds and surface water became a major concern. The affects of long-term exposure to low-level herbicide contamination is complex and needs to be carefully assessed when addressing environmental problems associated with herbicide use.

Two of the herbicides found contaminating watersheds and surface water are atrazine and acetochlor (David et al. 2003). Various studies have attempted to determine the toxicity, both acute and chronic, of these herbicides. Many of the toxicity studies use the more pure technical form of the herbicides (Meisner et al. 1992; Biradar and Rayburn 1995; Taets et al. 1998; Cheek et al. 1999; Crump 2002; Freeman and Rayburn 2004). One aspect that is often overlooked is that applied herbicides that eventually contaminate the environment are usually mixed with various classes of chemicals that may be more toxic than the labeled active ingredient (Mann et al. 2003).

Recent studies have indicated that the chemical additives in commercially available herbicides are toxic to aquatic organisms (Mann and Bidwell 2001; Haller and Stocker 2002; Lajmanovich et al. 2003). While these studies are informative, one factor is still not taken into account. Organisms in areas of agrochemical contamination will be exposed simultaneous to chemicals in the herbicide mixtures. A few studies have compared the toxicity of a herbicide with its formulations (Mann and Bidwell 1999), however such comparative studies are scarce.

The objective of this study was to compare the toxicity of the technical grade atrazine and acetochlor with their respective formulations. In addition, because

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atrazine and acetochlor are often mixed and applied together, the technical grade mix and a commercial formulation were compared. The purpose of this study was to determine if additives in commercial herbicides formulations result in an increase of cytotoxicity beyond the herbicide itself.

MATERIALS AND METHODS

Atrazine (CAS No. 1912-24-9) and acetochlor (CAS No. 34256-82-1) were purchased from Chem Services, Inc. The formulation containing atrazine was Atrazine 90DF produced by Monsanto. The formulation contained 86.5% atrazine, 3.6% related compounds and 9.9% inert compounds. The acetochlor formulation used was Harness by Monsanto. Harness contains 74.5% acetochlor and 25.2% unnamed ingredients. The mixed formulation of atrazine and acetochlor (HarnessXtra), also produced by Monsanto, contained 46.3% acetochlor, 18.3% atrazine and 35.4% unnamed ingredients. DegreeXtra also an atrazine-acetochlor mixture contains 29% acetochlor, 14.5% atrazine and 56.5% other ingredients. The commercial formulations were provided by Dr. D. Riechers, University of Illinois.

The micoplate mammalian cell toxicity assay of Sorensen et al. (2003) was used. The Chinese hamster ovary (CHO) cell line AS52 (subclone 11-4-8) was obtained from Dr. M. J. Plewa. CHO cells were cultured and maintained in complete F10 Hams media with 200 mM L-glutamine and 15% calf serum at 37°C and 5% CO_2 in a humidified chamber. At confluency cells were washed in 1X phosphate buffered saline (PBS) and detached from flask by trypsin treatment. Cells were centrifuged at 1200 rpm for 5 minutes. The supernatant was aspirated and the pellet resuspended in the residual media followed by the addition of 10 mL fresh media. A 1:200 cell dilution was made and cells were counted on a Coulter Counter Z Series (Coulter Electronics, Hileah, FL). A 3 x 10^4 cells/ml cell stock was prepared.

Assays were set-up in 96-well plates with appropriate volume of media and chemical stock to achieve desired test concentrations. 3000 cells were then added per well. Plate set-up included a first column blank and a second column negative control. Each plate contained four subsample wells per chemical concentration. After set-up plates were placed in a humidified chamber at 37°C and 5% CO_2. After 72 hours the cells were fixed in 50% methanol, stained with 1% crystal violet in 50% methanol and read on a Multiskan RC microplate reader at 595 nm. The percent of the negative control was calculated for each test concentration. This number represents the confluency of the cells grown in the presence of the test compound as compared to the unexposed control cells. Four replicate plates per chemical were completed. The average percent confluency of the control values were plotted in Sigma plot as a sigmoidal curve. The 50% confluency of the control values ($\%C_{1/2}$) were calculated for each replicate per treatment. An ANOVA (analysis of variance) was run on the $\%C_{1/2}$ values and an LSD (least

significant difference) analysis performed using SAS (version 8.2, SAS Institute Inc., Cary, NC, USA).

Two experiments were performed. In the first experiment, technical and field grade atrazine (90DF) and acetochlor (Harness) were examined. In addition, caffeine was used as a positive control. The molarity of each sample was determined based on the molecular weight of the specific compound and its formulation. The curves were compared as described above. In the second experiment, acetochlor was examined. In addition to technical and field grade acetochlor, mixtures of atrazine and acetochlor were also examined. Two different mixtures DegreeXtra and HarnessXtra were evaluated. The technical atrazine and acetochlor were mixed to mimic the ratios found in the field formulations. The molarity of acetochlor was the molarity used in comparison studies since all the samples contained acetochlor. The curves were compared as described above.

RESULTS AND DISCUSSION

All chemicals tested were found to result in well defined cytotoxicity curves. Upon regression analysis, the curve fit and the actual data was found to have an R value of 0.99 in all chemicals analyzed. This high correlation coefficient is indicative of the robust nature of the technique and also allows for accurate determination of the $\%C_{1/2}$ value.

In experiment 1, both atrazine and acetochlor were found to be more cytotoxic than caffeine (Figure 1, Table 1). The $\%C_{1/2}$ value for caffeine was 1.58 mM. This value is similar to the $\%C_{1/2}$ value reported for caffeine by Rayburn et al. (2001). The $\%C_{1/2}$ value of atrazine was one-third that of caffeine, while the $\%C_{1/2}$ of acetochlor was approximately one-thirtieth of caffeine (Table 1). Thus in experiment 1, the ranking of the toxicity was caffeine < atrazine < acetochlor. This ranking is in agreement with the various reported toxicities of atrazine and acetochlor (Weed Science Society of America 2002). In addition, the $\%C_{1/2}$ value for atrazine observed in this study was similar to the $\%C_{1/2}$ value for atrazine obtained by Sorensen et al. (2003). Therefore, the data presented here is consistent with previous reports.

With regards to field formulation versus technical grade, no noticeable differences were observed (Figure 1, Table 1). Upon comparing the curves of both atrazine and acetochlor, the field formulation was very similar to the technical grade chemical. The $\%C_{1/2}$ of the technical grade acetochlor was 0.05 mM while the $\%C_{1/2}$ of the field grade acetochlor was 0.04 mM. This difference was not statistically significant. Although Harness contained approximately 25% unnamed chemicals, the mixture did not result in increased toxicity at equivalent

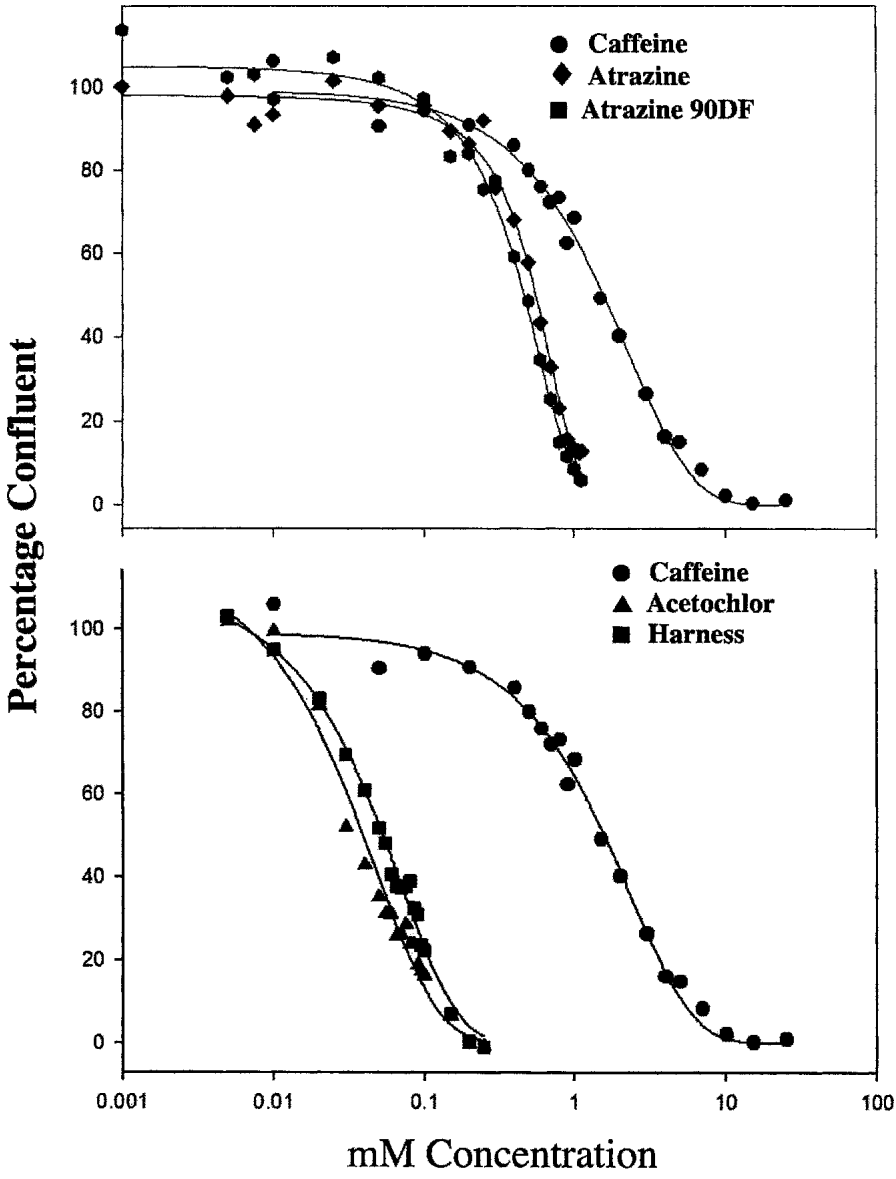

Figure 1. Cytotoxicity curves of atrazine and acetochlor with caffeine as the control chemical.

acetochlor concentrations. A similar pattern was seen with atrazine. The chemicals added to the field formulations had little if any affect on the cytotoxicity of the herbicides. The purpose of the second experiment was two-fold. The first purpose was to repeat the toxicity curves of acetochlor, both field and technical grade and compare their $\%C_{1/2}$ values (Table 2). The $\%C_{1/2}$ of technical grade acetochlor in experiment 1 was 0.05 mM. In experiment two, the $\%C_{1/2}$ of the technical grade acetochlor was an identical 0.05 mM. The same equivalent was noted with Harness as well. The $\%C_{1/2}$ was an identical 0.04 mM in both experiments. Despite the facts that one individual ran experiment 1 while a second individual ran experiment 2 and that experiment 2 was performed 6 months after experiment 1, the $\%C_{1/2}$ values were the same for common chemicals tested. These results attest to the robust nature of the cytotoxicity test as well as to its reproducibility.

Table 1. Cytotoxicty of acetochlor and atrazine as expressed by $\%C_{1/2}$ values.

Herbicide	Formulation	$\%C_{1/2}$*	R value
Acetochlor	Technical grade	0.05 mM[a]	0.99
Acetochlor	Harness	0.04 mM[a]	0.99
Atrazine	Technical grade	0.56 mM[b]	0.99
Atrazine	90DF	0.46 mM[b]	0.99
Caffeine	Technical grade	1.58 mM[c]	0.99

* $\%C_{1/2}$ values with the same letter are not significantly different at $p = 0.05$.

Table 2. Cytotoxicty of acetochlor and acetochlor-atrazine mixtures as expressed by $\%C_{1/2}$ values

Herbicide	Formulation	$\%C_{1/2}$*R value	
Acetochlor	Technical grade	0.05 mM[a]	0.99
Acetochlor	Harness	0.04 mM[a]	0.99
Acetochlor/Atrazine	HarnessXtra	0.04 mM[a]	0.99
Acetochlor/Atrazine	Technical grade HarnessXtra mixture	0.10 mM[b]	0.99
Acetochlor/Atrazine	DegreeXtra	0.15 mM[c]	0.99
Acetochlor/Atrazine	Technical grade DegreeXtra mixture	0.09 mM[b]	0.99

* $\%C_{1/2}$ values with the same letter are not significantly different at $p = 0.05$.

The second purpose of experiment 2 was to determine if field mixes of acetochlor and atrazine resulted in an increase in cytotoxicity. The HarnessXtra field formulation was observed to have the same $\%C_{1/2}$ (0.04 mM) as Harness. The $\%C_{1/2}$ values of the Harness and HarnesXtra were not significantly different from each other or from the technical grade acetochlor (Table 2, Figure 2). Thus mixing atrazine and having approximately 35% other chemicals did not increase

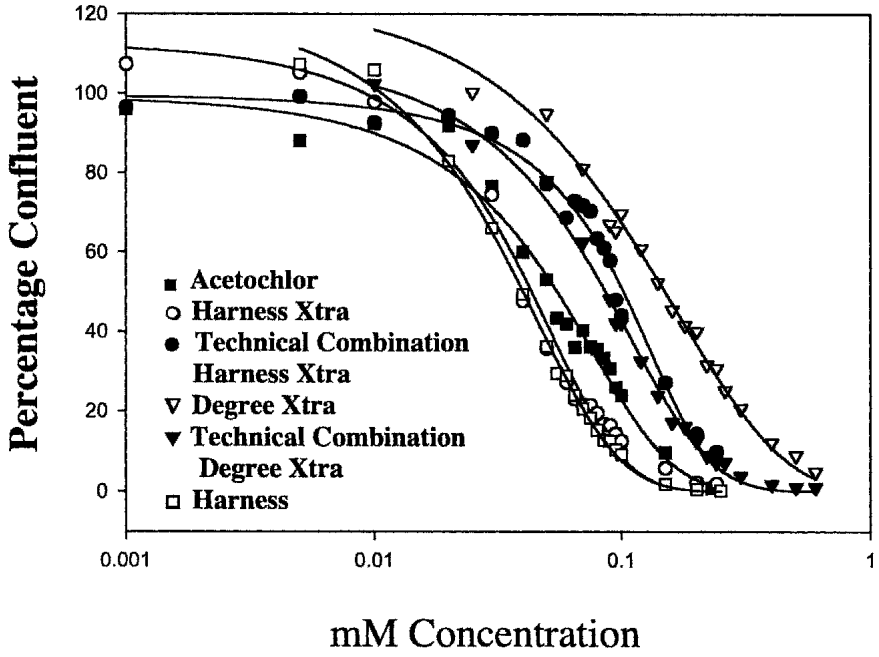

Figure 2. Cytotoxicity curves of acetochlor and acetochlor/atrazine mixtures.

the cytotoxicity of acetochlor. Again, the comparisons are made at the same acetochlor concentrations regardless of the added ingredients. The technical grade mixture of atrazine and acetochlor at HarnessXtra concentrations also gave no indication that mixing atrazine with acetochlor increased the overall cytotoxicity of the mixture. The technical grade DegreeXtra mixture gave results similar to the technical grade HarnessXtra concentrations. Again, no indications of increased toxicity were seen. Interestingly, DegreeXtra (field formulation) resulted in the most significant decrease in cytotoxicity. Harness Xtra was threetimes more cytotoxic than DegreeXtra. However, the technical grade mixtures of the two field formulation had similar $\%C_{1/2}$ values that were not statistically different at $p = 0.05$. These two technical grade mixtures have similar acetochlor to atrazine concentrations of approximately 50:50. The similarity in cytotoxicity is reflective of the similarity of the acetochlor to atrazine mixtures.

The decreased cytotoxicity of DegreeXtra is due to the formulation of the herbicide product. Although DegreeXtra is reported to contain 29% acetochlor and 14.5% atrazine, the herbicides are not completely available to the environment. According to DegreeXtra's label, the herbicide is encapsulated for controlled release. The release of the herbicide is affected by temperature. As the temperature rises, the herbicide is released. Exposure to 37°C for 72 hr does

result in herbicide release but the release is incomplete and thus results in a reduced $\%C_{1/2}$ due to the concentration of herbicide being less than predicted. With respect to environmental concerns, the delay released formulations appear to be performing as expected and not allowing a large flush of toxic concentrations of the herbicides to be released at one time into the environment.

In conclusion, the mammalian cellular toxicity test was found to be a highly reproducible technique for the assessment of herbicide cytotoxicity. By using this method, the field formulations were found to not be anymore cytotoxic than the technical grade chemicals indicating that the addition of chemicals to the herbicides does not increasing the cytotoxicity of the herbicides. Although this study used a mammalian cytotoxicity assay, the $\%C_{1/2}$ values obtained from this assay have been found to be highly correlated with the LD_{50} values observed in rat in vivo assays (Sorensen et al. 2003). Therefore while one cannot use the cytotoxicity assay to definitively determine the in vitro toxicity of contaminates to any one particular organism, the microplate mammalian cell toxicity assay provides a way to initially screen the toxicity of environmental contaminates.

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