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Relative Toxicity and Occurrence Patterns of Pesticide Mixtures in Streams Draining Agricultural Watersheds Dominated by Corn and Soybean Production

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Original Research

ABSTRACT

To evaluate the relative toxicity and the occurrence patterns of pesticide mixtures in streams draining agricultural watersheds, a 3-step approach was used. First, a landscape of interest was identified, defined, and isolated. Second, the relative toxicity of mixtures, on the basis of pesticide toxicity index scores, was compared with the relative toxicity of the highest individual pesticide, on the basis of highest toxicity quotient values. Third, occurrence patterns of pesticide mixtures were identified for use in follow-up mechanistic studies. The landscape of interest was identified as the corn and soybeans crop setting and concentrations of pesticides in streams within this crop setting were determined from US Geological Survey data. Pesticide toxicity index scores for individual samples were highest for the primary producers, *Pseudokirchneriella subcapitata* and *Lemna gibba*; with 95th percentile pesticide toxicity index scores of 4.7 and 1.9, respectively. The 95th percentile pesticide toxicity index score for *Daphnia magna* was 0.40 when a chronic sublethal endpoint was used. Pesticide toxicity index values were above the highest toxicity quotient values, indicating that consideration of mixtures does increase the estimated risk, but pesticide toxicity index scores were generally within a factor of 2 of highest toxicity quotient values, indicating that the increased risk is not large for most samples. Pesticide toxicity index scores tended to be dominated by individual pesticides and simple mixtures. Two different prioritization strategies were used to identify important mixtures for further study on the basis of potential effects on *P. subcapitata*. Both techniques decreased the complexity of the pesticide mixtures to consider by reducing the number of components within the identified mixtures as well as identifying a few specific combinations that constitute the majority of mixtures within the sample. Nearly all important pesticides for *P. subcapitata* were herbicides from 2 chemical classes: Acetanilide and triazine herbicides.

Keywords: Pesticide toxicity index Mixtures Hazard quotients Corn Soybean

INTRODUCTION

Assessment of pesticide occurrence in streams and groundwater throughout the United States shows that pesticides are frequently detectable in streams with substantial agricultural or urban land use in their watersheds and that pesticides most often occur as mixtures of pesticides (Gilliom et al. 1999, 2006). Additionally, a large body of research has accumulated that demonstrates that the presence of multiple toxicants generally results in greater toxicity than any of the individual components (Altenburger et al. 2003; Lydy et al. 2004). In fact, several studies have shown that a mixture of chemicals below their lowest observed effect concentration can cause a toxic effect (Broderius and Kahl 1985; Altenburger et al. 2000). Together, these findings have raised concerns in regard to the potential ecological effects of pesticide mixtures.

Despite these concerns, limited work has been conducted for specific land use and crop settings, herein referred to as "pesticide usage landscapes," that evaluates whether or not the environmental occurrence of pesticide mixtures pose significantly more risk to aquatic organisms than would be predicted by evaluating pesticides individually. Although some degree of increased risk is almost certainly caused by the presence of more than 1 stressor in a system, the magnitude of increased risk needs to be quantified to better evaluate water quality at a watershed level. This study takes a 3-step approach to evaluate the potential risks posed by mixtures and includes identifying and isolating a pesticide usage landscape for study, estimating the relative toxicity of mixtures compared with the highest individual pesticide in a sample, and evaluating occurrence patterns of pesticide mixtures for use in follow-up mechanistic studies.

Pesticide usage landscape

To reduce the complexity of evaluating pesticide mixtures, the scope of this study was limited to considering a single–pesticide usage landscape. Pesticide usage landscapes are closely related to land use or crop pattern descriptions but signify that the land use designation was tightly defined in relation to pesticide use practices and only analyzed over a specific temporal range to determine the effects of specific pesticide usage patterns. Focusing on a pesticide usage landscape allows simplification of the analysis of potential pesticide mixtures and provides results that are directly applicable to a particular geographic area. In our current work, we defined the pesticide usage landscape to match the US Geological Survey (USGS)–defined agricultural row crop category of corn and soybean. The corn and soybean crop group is the largest US crop setting and accounts for 32% of row crop production or 33 million ha (Gilliom and Thelin

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Table 1. Target species, test conditions, and effect endpoints

Common name	Species	Test duration	Endpoint[a]
Green algae	*Pseudokirchneriella subcapitata*	96 h	50% reduction in growth and reproduction (absorbance)
Duckweed	*Lemna gibba*	14 d	50% reduction in reproduction (frond count)
Daphnia	*Daphnia magna*	21 d	Life cycle, LOEC
Daphnia	*D. magna*	48 h	50% mortality or immobility
Bluegill	*Lepomis macrochirus*	96 h	50% mortality

[a] LOEC = lowest observed effect concentration.

1997). We only evaluated stream sites with watersheds dominated by this crop setting and only analyzed pesticide data throughout the growing season (full details follow in *Methods*).

Pesticide toxicity index

The pesticide toxicity index (PTI) was the approach chosen to address the relative risk of mixtures (Munn and Gilliom 2001). The PTI provides an estimate of the relative toxicity of a pesticide mixture on the basis of the concentration–addition model of toxicity in a similar way as a hazard quotient is used in standard risk assessments. Before PTI determinations, toxicity quotients (TQs) must be calculated by dividing individual pesticide concentrations for each environmental sample by the chosen toxicity value (e.g., median lethal or effect concentration, LC50 or EC50, or the lowest observed effect concentration, LOEC) for that pesticide. The PTI score is then calculated for a given sample by summing all of the TQs for that sample,

$$\mathrm{PTI} = \sum_{i=1}^{n} \frac{\mathrm{C}_i}{\mathrm{TC}_{x,j}} = \sum \mathrm{TQ}$$

where C_i is the concentration of pesticide i, $TC_{x,i}$ is the toxic concentration of pesticide i for species x (e.g., LC50, EC50, or LOEC). To address the concern of increased risk because of exposure to pesticide mixtures compared with the risk that would be estimated from exposure to a single pesticide, PTI scores were compared back to the highest TQ (HTQ) for a sample. Because TQs are mathematically equivalent to hazard quotients, the HTQ represents the greatest hazard posed by an individual pesticide.

The PTI is mathematically similar to the toxic unit (TU) approach that is commonly used as an evaluation tool for pesticide mixtures. In the TU approach, a TU value for a single pesticide is calculated in the same manner as a TQ and the sum TU is mathematically equivalent to PTI. Thus, the PTI approach is grounded in mixture theory. However, the TU approach requires specific assumptions, such as similar modes of toxic action for the test chemicals and that the TU should be calculated with toxicity data that were generated with the use of exact toxicological methods (Altenburger et al. 2003; Lydy et al. 2004). In contrast, the PTI is defined as a ranking index (Munn and Gilliom 2001). As such, no specific species or environmental effects are assigned to specific PTI values. Thus, chemicals with dissimilar modes of toxic action and toxicity values generated by different methods are used to calculate a PTI. Pesticide toxicity indices can be calculated for an individual species, similarly to a TU, or they can be calculated with toxicity values generated from a larger taxonomic group. In this study, 4 species (e.g., *Pseudokirchneriella subcapitata*, *Lemna gibba*, *Daphnia magna*, and *Lepomis macrochirus*) and 5 endpoints (growth, reproduction, life cycle test, mortality, and immobility) were evaluated (Table 1).

Occurrence frequency of pesticide mixtures

Although several mixture modeling techniques such as independent action (IA) (Backhaus et al. 2000; Faust et al. 2000, 2003), concentration addition (CA) (Altenburger et al. 2000; Faust et al. 2001; Junghans et al. 2003), and IA/CA hybrid approaches (Altenburger et al. 2004; Olmstead and LeBlanc 2005) have been suggested and supported by some mechanistic studies, many uncertainties still exist. For instance, although the techniques can be selected on the basis of mode of toxic action, the degree of inclusiveness of which mode of toxic action is most appropriate has not been well defined (Altenburger et al. 2003; Lydy et al. 2004). Additionally, several studies have shown that pesticide combinations exhibit toxicity greater than that predicted by the models (Belden and Lydy 2000; Denton et al. 2003; Lydy et al. 2004; Belden and Lydy 2006). In regard to the general use of the models, uncertainties might remain indefinitely. Thus, it is important for investigators to conduct toxicity tests on frequently occurring mixtures until models can be fully developed and validated for the wide range of mixtures that might be important.

Selection of the most important mixtures is a complicated task because of the complexity of most environmental samples. Analytical methods used for the detection of pesticides in surface water often have very low detection limits (parts per trillion) and frequently indicate the presence of a wide array of pesticides (Gilliom et al. 1999, 2006). On the basis of simple occurrence, the number of unique mixtures is very large, and approaches are needed to identify the particular mixtures most likely to be of concern. Even in human health risk assessments, in which toxicity to only 1 species is to be predicted, the toxicological significance of complex mixtures has been difficult to address. To simplify the task, an approach referred to as the "top 10 approach" has been used (Feron et al. 1998). In this approach, the complex mixture is simplified to include only the top 10 (or similar number) components that are the most likely to cause an environmental impact. Censoring (omitting) individual toxicants that occur in mixtures is accomplished on the basis of a combination of the toxicity of each pesticide and the proportion of the mixture that it composes. The reduced mixture is then studied in more detail and evaluated in regard to potential human response.

Table 2. List of sites that matched this study's criteria for a corn–soybean crop system

Site	Sampling dates	Nr of samples[a]
Lost River near Leipsic, IN, USA	1 May–8 Aug 1994	6
Clifty Creek at County Road 1150 East, Hartsville, IN	12 Apr–17 Aug 1994	7
Muscatatuck River near Deputy, IN	13 Apr–30 Aug 1994	8
East Fork White River at Shoals, IN	19 Apr–26 Aug 1994	8
Big Walnut Creek at County Road 700 West, Reelsville, IN	12 May 1994–17 Apr 1995	7
Maumee River at Waterville, OH, USA	17 Apr–13 Aug 1997	10
St Joseph River near Newville, IN	16 Apr–12 Aug 1997	13
Auglaize River near Fort Jennings, OH	15 Apr–11 Aug 1997	14
Little River Ditch nr 1 near Morehouse, MO, USA	1 Apr–26 Aug 1997	20
Little Cobb River near Beauford, MN, USA	8 Apr–26 Aug 1997	21
Minnesota River near Jordan, MN	22 Apr–13 Aug 1997	7
Mississippi River below Lock and Dam nr 2, Hastings, MN	13 Apr–30 Aug 1997	7
Iowa River near Rowan, IA, USA	3 Apr–26 Aug 1997	18
Wolf Creek near Dysart, IA	2 Apr–25 Aug 1997	18
Iowa River at Wapello, IA	10 Apr–28 Aug 1997	16
La Moine River at Colmar, IL, USA	1 Apr–26 Aug 1997	26
Sangamon River at Monticello, IL	3 May 1997–27 Apr 1998	32

[a] Number of samples used from each site and the earliest sampling date. All samples were collected within a year of the earliest sampling date.

In this study, 2 different approaches, both similar in concept to the top 10 approach, were used to evaluate and censor mixtures found in streams. The 1st approach was designed as a tool for initial screening. In this approach, each sample within the database was censored according to toxicologically based values for each detected pesticide, or levels of interest (LOIs), thus reducing the complexity of each detected mixture by including only those pesticides most likely to contribute to toxicity. The LOI chosen in this study was 0.5% of the EC50. The LOI was set at 0.5% of the EC50 on the basis of preliminary analysis of the dataset that indicated fewer than 20 pesticides would be present in the samples. Assuming that a PTI value of 0.10 would warrant concern, 20 components would all have to be present at 0.5% or higher of their EC50 value (or 0.005 TQs) to achieve or cross that threshold. Thus, only pesticides that occurred above 0.5% of the EC50 were considered as a component of the mixture for a given sample. Because of differences in toxicity, the resulting screened database is species dependent. In this study, this approach was referred to as the LOI approach.

The 2nd approach was designed as a tool for evaluating essential components of known mixtures. In this approach, censoring was conducted on the basis of each component's contribution to the PTI. Some pesticides in a sample invariably contributed much more to the PTI than others. To simplify the mixtures, only mixture components that contributed greater than 10% of the PTI value were considered mixture components. This approach was referred to as the primary constituent (PC) approach.

To demonstrate these techniques, the frequency of occurrence for mixtures following screening is presented for *P. subcapitata*. This species was chosen for further analyses because its PTI index was the highest of the species tested indicating it is the organism at the greatest risk within this pesticide usage landscape.

METHODS

Pesticide usage landscape

Pesticide concentration data were obtained from a USGS database that contains the results from the National Water Quality Assessment Program. The program began in 1991, focusing on water quality in 51 major river basins and aquifer systems covering more than one-half of the land area of the conterminous United States (USGS 1999). From this database, specific stream sites were selected on the basis of the following parameters to insure that the pesticide usage landscape was consistent throughout sites and corresponded to the corn–soybean crop category.

1. Watersheds must be greater than 50% agricultural and less than 5% urban land use.
2. Cropland in the watersheds must have greater than 20% coverage in corn and greater than 20% coverage in soybean.
3. Analytical data from 2 primary analytical methods, GC/MS and high-performance liquid chromatography, had to be available for all samples to ensure consistent data for the pesticides of interest.

The above parameters resulted in the selection of 17 sites on streams located primarily in the Corn Belt region of the United States (Table 2). For each site, the data for the most complete year of sampling on record were used. The beginning of sampling at the sites ranged from 12 April 1994 to 27 April 1998. Only data collected from April

through August were used to coincide with the highest pesticide application rates and subsequent highest runoff concentrations. All data collected for the 17 sites during this seasonal period were used in the analysis, such that for some sites, only 1 sample was considered for each month of the year, whereas other sites had multiple samples considered per month. By this approach, 238 samples were included in the analysis. Although this introduces site bias in the analysis, it increases the number of samples available and increases the odds of including the full range of pesticide concentrations that occur from "pulsing" of concentrations as a result of application and rain events.

The USGS pesticide analyses included 83 compounds, including both pesticides and degradation products (Werner et al. 1996; Zaugg et al. 1995). For the 17 sites included in this study, 45 pesticides and 3 degradation products were detected. Standard toxicological values were not available for the degradation products dichlorodiphenyldichloroethylene, dacthal monoacid, and deethylatrazine; therefore, only the 42 pesticides listed in Table 3 were further evaluated in this study.

Toxicological concentrations

Endpoints were chosen to optimize taxonomic diversity with the use of standard bioassay data that were available for most pesticides because of registration requirements. Furthermore, all chosen species and endpoints have defined methods outlined for toxicity testing, allowing the data to be relatively consistent (Table 1). Toxicity data were primarily obtained through the US Environmental Protection Agency's (USEPA's) Pesticide Ecotoxicity Database (Office of Pesticide Programs, contact B. Montague). If values were unavailable, the USEPA ECOTOX database (http://www.epa.gov/ecotox/, Office of Research and Development, and the National Health and Environmental Effects Research Laboratory's Mid-Continent Ecology Division) was checked. If data were not available in either database for a pesticide, species, or endpoint, a related species or similar endpoint was used. An attempt was made to obtain toxicity values for all pesticides detected above reporting limits in the database (Table 3). In cases in which multiple values were available for a pesticide, species, or endpoint, preference was given to studies conducted with active ingredient above 85% purity. If multiple values still were found, the median value was used. Table 3 lists the EC50 or LC50 values for all 4 species.

Calculation of PTIs and HTQs

The PTI values for each sample were calculated with Equation 1, sample concentrations from the described database, and toxicity values from Table 3. In some cases, toxicity values were not available for a particular species; therefore, the PTI values for that species were calculated after excluding those pesticides. The HTQ for an individual pesticide was determined by dividing the concentration of each pesticide in a sample by the toxic concentration for that pesticide and selecting the highest value. Percentile ranks were calculated on the basis of the entire dataset.

Contribution of individual pesticides and simple mixtures to PTI scores

To evaluate the contribution of individual pesticides and simple mixtures (binary and tertiary) to PTI scores, the percent contribution of each pesticide to the PTI score for that sample was calculated by dividing the TQ for each pesticide by the PTI for the sample multiplied by 100. Isolating the largest value for each sample identified the pesticide with the highest percent contribution for each sample, and identifying the largest 2 or the largest 3 values isolated binary and tertiary mixtures. Only samples with a PTI greater than 0.10 were included in the analyses. The 0.10 censoring level was established to prevent bias in the analyses from samples containing low concentrations of pesticides.

Prioritizing mixtures with the LOI approach

The type and presence of mixtures remaining from toxicological censoring was species dependent; thus, the current analysis was conducted for *P. subcapitata*. Pesticide concentrations for each sample were censored with a LOI value set to 0.5% of the EC50 (Table 3). If the concentration in the sample was below the LOI, then the concentration was no longer considered for further analyses. For a few pesticides, the analytical reporting limit was higher than the LOI. In these cases, all reported values were considered.

Prioritizing mixtures with the PC approach

In the 2nd approach, censoring was performed according to the contribution of each pesticide to the PTI score for a sample. Any pesticide that did not have a TQ that contributed greater than 10% of the PTI score was censored from the mixture. Samples with very low PTI scores can be biased in regard to the number of reported components because 10% of the PTI score (the censoring level) might be well below the reporting limits. Thus, only samples with a PTI greater than 0.10 were used for these analyses (148 of the 238 samples analyzed).

Evaluation of mixtures

The censored databases were evaluated to determine the number of pesticides of interest that were present in samples and the most common mixtures that were present in the samples. Samples were retained as individual units so that actual exposure patterns could be analyzed.

RESULTS

Occurrence of mixtures and species comparisons

Pseudokirchneriella subcapitata had the highest PTI scores, followed by *L. gibba* and *D. magna* chronic. The PTI evaluation for these 3 species/endpoints indicated higher relative toxicity, with a 95th percentile PTI score of 0.40 or greater (Table 4), compared with *D. magna* acute and *L. macrochirus*, for which the 95th percentile PTI scores were 0.05 and 0.02, respectively.

Relative toxicity of mixtures compared with individual pesticides

For *P. subcapitata* and *L. gibba*, evaluation of mixtures caused an increase in relative risk, as indicated by PTI scores that were 162% to 200% larger than the HTQ for *P. subcapitata* and 150% to 190% larger than the HTQ for *L. gibba* (Table 4). For both *D. magna* endpoints, pesticide mixtures posed relatively less increase in risk, with PTI scores that ranged from 114% to 141% of the HTQ value. For *L. macrochirus*, mixtures tended to increase the relative risk, with PTI values that were 136% to 160% of the HTQ values; however, the PTI and HTQ values for fish were relatively low (<0.02).

Table 3. Pesticides found in samples within the database selected for corn–soybean crop usage. Reporting limits indicate the lowest toxic concentration reported in the US Geological Survey database. For each species, the median lethal or effect concentration (LC50 or EC50, respectively) is presented when data were available. — = data not available; CAS = Chemical Abstracts Service

Pesticide	CAS	Reporting limit (μg/L)	Median concentration (μg/L)				
			Pseudokirchneriella subcapitata	Duckweed	*Daphnia* acute	*Daphnia* chronic	Bluegill
2,4-D	94-75-7	0.16	33,000	700	25,000	56,000	220,000
Acetochlor	34256-82-1	0.006	1.4	3.4	8,200	1,200	1,500
Acifluorfen	50594-66-6	0.09	270	380	28,100	—	47,000
Alachlor	15972-60-8	0.0045	1.6	340	21,000	—	4,300
Atrazine	1912-24-9	0.007	53	40	6,900	250	24,000
Azinphos-methyl	86-50-0	0.05	—	—	1.1	0.40	4.5
Benfluralin	1861-40-1	0.01	2,500	200,000	100	31	330
Bentazon	25057-89-0	0.05	4,500	5,400	125,000	—	610,000
Bromoxynil	1689-84-5	0.07	7,800	8,100	19,000	40	4,000
Butylate	2008-41-5	0.002	6,500	4,100	12,000	—	6,900
Carbaryl	63-25-2	0.041	1,100	1,000,000	5.6	6.0	9,500
Carbofuran	1563-66-2	0.02	270,000	—	29	27	640
Chlorpyrifos	2921-88-2	0.005	—	—	0.9	0.08	3.0
Cyanazine	21725-46-2	0.018	13	64	46,000	—	23,000
Dacthal	1861-32-1	0.003	12,000	11,000	100,000	—	6,700
Diazinon	333-41-5	0.005	3,700	100,000	0.96	0.32	170
Dicamba	1918-00-9	0.11	3,700	3,300	110,000	—	140,000
Dieldrin	60-57-1	0.0048	100	1,000,000	250	—	4.4
Diuron	330-54-1	0.12	2.4	25	8,400	200	3,000
EPTC	759-94-4	0.002	1,400	5,600	7,000	1,300	18,000
Ethoprop	13194-48-4	0.005	—	—	93	3.9	300
Fluometuron	2164-17-2	0.06	170	410	10,000	—	45,000
Fonofos	944-22-9	0.003	100	—	12	0.64	29
gamma-HCH	58-89-9	0.004	—	—	6,500	110	25
Linuron	330-55-2	0.035	67	27	270	240	9,600
Malathion	121-75-5	0.027	—	—	1.0	0.10	62
Methylparathion	298-00-0	0.006	15,000	—	0.14	0.85	4,400
Metolachlor	51218-45-2	0.013	10	46	66,000	3,800	10,000
Metribuzin	21087-64-9	0.006	8.1	160	4,200	2,600	76,000
Molinate	2212-67-1	0.004	220	3,300	4,700	900	23,000
Napropamide	15299-99-7	0.007	3,400	—	20,000	—	12,000
Pendimethalin	40487-42-1	0.022	5.4	13	280	17	200
Prometon	1610-18-0	0.018	98	—	26,000	680	42,000
Propachlor	1918-16-7	0.01	10,000	5.2	7,800	—	1,400
Propanil	709-98-8	0.011	29	110	6,700	120	5,400
Propargite	2312-35-8	0.023	66	75,000	91	—	99

Table 3. Continued

Pesticide	CAS	Reporting limit (µg/L)	Median concentration (µg/L)				
			Pseudokirchneriella subcapitata	Duckweed	*Daphnia* acute	*Daphnia* chronic	Bluegill
Simazine	122-34-9	0.011	100	140	1,100	2,500	16,000
Tebuthiuron	34014-18-1	0.016	100	140	300,000	44,000	110,000
Terbufos	13071-79-9	0.017	—	—	0.35	0.076	0.83
Thiobencarb	28249-77-6	0.0048	17	770	100	2.0	2,500
Triclopyr	55335-06-3	0.25	18,000	8,200	130,000	—	148,000
Trifluralin	1582-09-8	0.009	670	44	560	51	8,400

Contribution of individual pesticides and simple mixtures to P. subcapitata *PTI scores*

The PTI scores were often dominated by the TQs of a few pesticides found within the sample. For example, in 29% of the samples, a single pesticide accounted for more than 60% of the PTI score, and in 7% of the samples, a single pesticide accounted for more than 80% of the PTI score. Simple mixtures were even more dominating. In 37% of samples, binary mixtures accounted for more than 80% of the PTI score, and in 86% of samples, tertiary mixtures accounted for more than 80% of the PTI score.

Complexity of mixtures

Every sample in the database contained a pesticide mixture when evaluated simply on the basis of detections above the reporting limits (no censoring). Furthermore, if samples were censored with a nominal value of 0.01 µg/L to prevent bias because of reporting limit variations, all samples still contained a pesticide mixture. However, if the pesticide concentrations were censored with *P. subcapitata* toxicological data, 220 of 238 samples contained a mixture by the LOI approach, and 130 of 238 samples contained a mixture by the PC approach.

Without censoring or nominal censoring, the typical mixture also contained a high number of components. However, if the pesticide concentrations were censored with *P. subcapitata* toxicological data via the LOI or PC approaches, the number of components within mixtures was reduced (Figure 1).

Important mixtures for P. subcapitata

The most common 16 mixtures for *P. subcapitata* after LOI censoring composed 84% of the LOI mixtures (Table 5). Nearly 70% of LOI mixtures were composed entirely of acetanilide herbicides (acetochlor, alachlor, and metolachlor) and triazine herbicides (atrazine, cyanazine, metribuzin, and simazine). A few pesticides occurred very frequently above their LOI in samples that contained LOI mixtures, including metolachlor (99%), acetochlor (82%), atrazine (72%), alachlor (71%), cyanazine (59%), metribuzin (24%), diuron (16%), pendimethalin (15%), and simazine (7.7%). All other pesticides occurred in less than 2% of samples that contained a LOI mixture.

The most common 15 mixtures for *P. subcapitata* from PC censoring accounted for 94% of all PC mixtures (Table 6). Acetanilide herbicide–only mixtures accounted for 40% of all PC mixtures, and mixtures composed of acetanilide, triazine

Table 4. Pesticide toxicity index (PTI, all mixture components) scores and the highest toxicity quotient (HTQ) for green algae (*Pseudokirchneriella subcapitata*, median effect concentration [EC50] for growth and reproduction), duckweed (*Lemna gibba*, EC50 for reproduction), *Daphnia* (*D. magna*, lowest observed effect concentration for life cycle assessment; EC50 or median lethal concentration [LC50] for lethality or mobility), and bluegill *(Lepomis macrochirus*, LC50). $n = 238$

Species		Percentile				
		Mean	50th	75th	90th	95th
P. subcapitata	HTQ	0.46	0.07	0.35	1.3	2.9
	PTI	0.86	0.14	0.59	2.2	4.7
Duckweed	HTQ	0.19	0.03	0.14	0.54	1.0
	PTI	0.31	0.05	0.21	0.89	1.9
Daphnia (chronic)	HTQ	0.080	0.0055	0.048	0.12	0.35
	PTI	0.091	0.0062	0.058	0.17	0.40
Daphnia (acute)	HTQ	0.014	>0.001	0.0047	0.018	0.042
	PTI	0.016	>0.001	0.0060	0.024	0.048
Bluegill	HTQ	0.0022	>0.001	0.0013	0.0038	0.010
	PTI	0.0030	>0.001	0.0018	0.0069	0.016

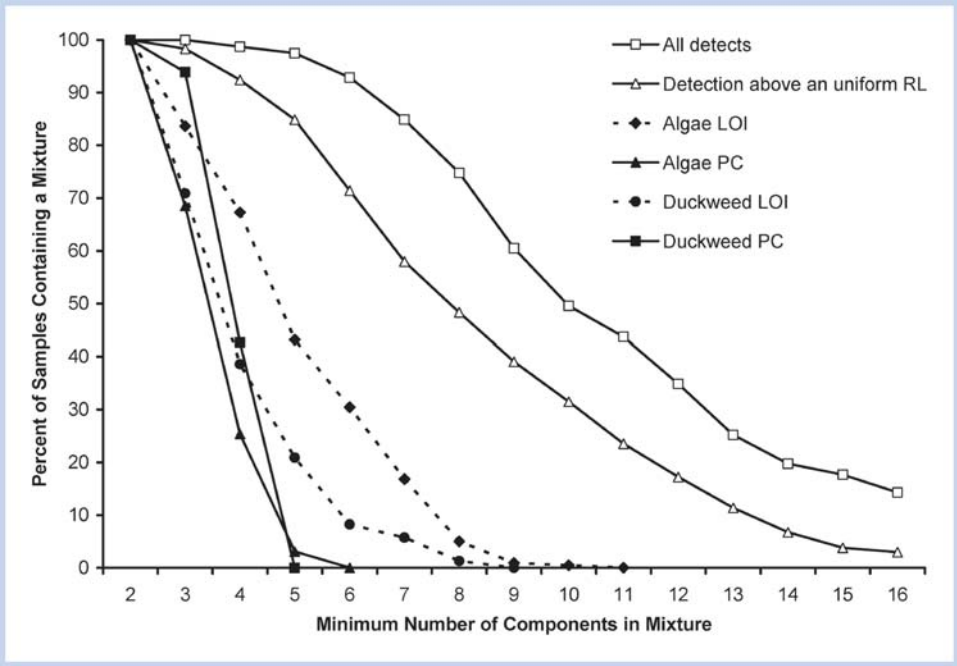

Figure 1. Percentage of samples containing a mixture are presented on the basis of the minimum number of components in the mixture for each censoring technique. Detection above a uniform reporting limit (RL) was based on the standard 0.01 μg/L cutoff. Level of interest (LOI) censoring used 0.5% of the median effect concentration (EC50) as the cutoff value and primary constituent (PC) censoring used 10% of the pesticide toxicity index score as the cutoff value.

Table 5. Most commonly occurring mixtures in surface water samples collected from a corn–soybean pesticide landscape on the basis of toxicological censoring with a level of interest (LOI) approach for green algae (*Pseudokirchneriella subcapitata)*. Mixtures with an occurrence frequency greater than 2% are listed. Total samples in the analysis were 238, 220 of which contained mixtures of at least 2 components above their LOI value. Listed mixtures account for 84% of mixtures found in samples

Mixture components[a]									Nr of pesticides in mixture	Frequency of samples containing a mixture (%)	Cumulative frequency (%)
Acet	Alac	Atra	Cyan	Diur	Metol	Metri	Pend	Sim			
X					X				2	14	14
	X	X	X		X				4	9.5	24
X	X	X	X		X				5	8.6	32
X	X				X				3	7.7	40
X	X	X	X		X		X		6	5	45
X		X	X		X				4	5	50
X	X	X			X				4	5	55
X	X	X	X		X	X			6	4.5	59
X	X	X	X		X	X	X		7	4.1	63
	X	X			X				3	4.1	68
X	X	X	X	X	X	X			7	3.6	71
X	X	X	X		X	X		X	7	2.7	74
X	X	X	X	X	X				6	2.7	77
X	X				X	X			4	2.3	79
X		X	X	X	X				5	2.3	81
X		X			X				3	2.3	83

[a] Acet = acetochlor; Alac = alachlor; Atra = atrazine; Cyan = cyanazine; Diur = diuron; Metol = metolachlor; Metri = metribuzin; Pend = pendamethalin; Sim = simazine.

Table 6. Most commonly occurring mixtures in surface water samples collected from a corn–soybean pesticide landscape on the basis of toxicological censoring with a principle constituent approach for green algae (*Pseudokirchneriella subcapitata)*. Mixtures that occurred at a frequency of greater than 2% are listed. Total samples in the analysis were 238, 130 of which met the criteria and are included in this analysis. Listed results account for 94% of mixture containing samples

Mixture components[a]							Nr of pesticides in mixture	Frequency of samples containing a mixture (%)	Cumulative frequency (%)
Acet	Alac	Atra	Cyan	Diur	Metol	Metri			
X					X		2	20	14
	X	X			X		3	14	34
X	X				X		3	12	46
X	X	X			X		4	6.9	53
X		X			X		3	6.2	59
	X	X	X		X		4	6.2	65
	X				X		2	5.4	71
X		X	X				3	4.6	75
X		X	X		X		4	3.8	79
X			X		X		3	3.1	82
X	X	X	X		X		5	2.3	85
X	X				X	X	4	2.3	87
X	X			X	X		4	2.3	89
X	X						2	2.3	91
		X	X		X		3	2.3	94

[a] Acet = acetochlor; Alac = alachlor; Atra = atrazine; Cyan = cyanazine; Diur = diuron; Metol = metolachlor; Metri = metribuzin.

herbicides, or both account for 89% of all PC mixtures. Common components of PC mixtures included metolachlor (90%), acetochlor (70%), alachlor (71%), atrazine (50%), cyanazine (25%), diuron (4.6%), and metribuzin (3.1%). All other pesticides occurred in less than 2% of samples that contained a PC mixture.

DISCUSSION

Relative toxicity of pesticide mixtures

Primary producers, as represented by *P. subcapitata* and *L. gibba*, are at the greatest and most frequent risk because of pesticide exposure within this pesticide usage landscape, as indicated by the magnitude of sample PTI scores (Table 4). Invertebrates could also be at risk on the basis of chronic toxicity to *D. magna*, and more limited risk was found for acute toxicity to *D. magna* and *L. macrochirus*, although the sample-based approach might under- or overestimate actual potential for effects on a stream. These relative sensitivities were not unexpected because herbicides were the most common pesticides in these agricultural areas (Gilliom et al. 2006). Similar results were also found in a previous study that used a toxic index approach. Battaglin and Fairchild (2002) reported that the risk for green algae and duckweed was higher than for bluegill and frogs in Midwestern US streams.

It should be noted that the relative PTI scores might be skewed by the endpoints evaluated. The 3 species endpoints of greatest concern—*P. subcapitata* growth and reproduction, *L. gibba* reproduction, and *D. magna* life cycle assessments—all had chronic sublethal endpoints, whereas the *L. macrochirus* and *D. magna* acute, which had the lowest PTI scores, were evaluated on the basis of acute lethal or near lethal endpoints, such as mobility. Ideally all PTI scores would also be calculated on the basis of chronic data; however, adequate datasets were not available. Regardless, because the PTI scores for *L. macrochirus* and *D. magna* acute were more than an order of magnitude lower than the scores for *P. subcapitata* and *L. gibba*, it is likely that the primary producers are at the most risk over the longest periods of time within this pesticide usage landscape, regardless of differences in endpoint sensitivity.

Increased risk by consideration of mixtures

When toxicity rankings (PTI scores) for mixtures were compared with rankings for individual pesticides (HTQ values) in the same samples, increased risk was noted for all species in the mixture exposures. However, PTI scores generally were less than 2-fold greater than the HTQ values. The dose–response curves of many pesticides are steep and a 2-fold difference can represent the difference in concentration between a no-observed-effect concentration and the EC50 (e.g., see Belden and Lydy 2006). However, in terms of some risk assessment applications, uncertainties in EC50s on the basis of intraspecies and interspecies variability in toxicity and uncertainties in estimating potential environmental concentrations could be much larger than a factor of 2. Thus, the relative importance of considering mixtures in this landscape depends on the goals and allowable uncertainties built into the risk assessment process.

Contribution of individual pesticides and simple mixtures

As could be ascertained by the relatively small differences between PTI and HTQ values (generally within a factor of 2), the PTI value for many samples was primarily attributable to a single pesticide and most were dominated by a simple mixture (binary or tertiary). Very few samples contained complex mixtures in which all pesticides occurred at concentrations that would cause a similar contribution to toxicity (equipotent mixtures). These results suggest that understanding the interactions of simple mixtures is very important in estimating the environmental risk of pesticides. Few studies to our knowledge have reported the contributions of specific pesticides in relation to the total mixture. Thus, it is unclear if this finding is specific to the pesticide landscape investigated or a common pattern of contamination. However, the dominant role of an individual pesticide was also reported in a recent study in which field-collected sediments in the San Joaquin Valley of California, USA, were found to be primarily attributable to a single pyrethroid insecticide, although several other pyrethroids and organochlorine insecticides were also present in the sediments (Weston et al. 2005).

In this study, the contamination patterns found for *P. subcapitata* indicated that most of the PTI score was composed of pesticides within the acetanilide and triazine herbicides classes. These patterns are not surprising because these 2 classes are among the heaviest used, are the most frequently occurring pesticides in the samples evaluated, and were the contaminants that had the highest concentrations. In fact, the 2 groups were the most commonly occurring pesticides in agricultural regions of the United States (Gilliom et al. 2006).

An implication of assuming that a limited number of pesticides and pesticide groups tend to dominate the potential risk to a particular species within this pesticide usage landscape is that simplification of the risk assessment process might be possible. If the mixtures can be considered simple mixtures, the risk assessment process could be easier because actual testing of important mixtures could be conducted. However, predictability of toxicity might not be any easier for simple mixtures than for multiple-component mixtures. Although a number of studies have suggested that mixtures with a large number of components tend to follow predictive models (Altenburger et al. 2003; Lydy et al. 2004), the toxicity of several binary mixtures has been reported to deviate from modeling methods (Lydy et al. 2004; Belden and Lydy 2006), especially for mixtures containing organophosphate insecticides. The potential dominance of mixtures by a few compounds indicates that continued testing of binary combinations, especially those in different classes, is important.

Complexity of mixtures

The approaches described in this paper reduced the number of mixtures and the number of components per mixture to those with the greatest likelihood of toxicological significance. For example, in the *P. subcapitata* data, 7 of the top 8 commonly occurring mixtures based on LOI analysis (Table 5) account for 50% of all LOI mixtures and comprise only 5 components from 2 herbicide classes (acetanilide and triazine herbicides). The PC analysis confirms and strengthens these trends. Combinations of the same 5 components account for 89% of PC mixtures. In contrast, analyses with no censoring or nominal censoring (0.01 g/L for each analyte) resulted in a large number of components and mixtures, but with many expected to have little toxicological significance.

The next step in gaining knowledge of potential toxicity to *P. subcapitata* of mixtures in streams with watersheds dominated by corn and soybean production is to understand the joint action within and between both classes of major herbicides. Previous research by Junghans et al. (2003) and Faust et al. (2001) has demonstrated that the algal toxicity of acetanilide herbicide mixtures and triazine herbicide mixtures, respectively, are best described by concentration addition models. However, to our knowledge, the joint toxicity of combinations of these herbicide groups has not been addressed with modeling techniques. The final step in gaining predictive knowledge is to perform the experimentation necessary to test specific mixtures. By reducing the number of specific mixtures to be tested to fewer than 10 and the components to fewer than 6, the project becomes feasible in the laboratory.

LOI determination of mixtures

By design, the LOI approach is useful as a screening tool. It limits the components of a sample by censoring pesticide detections that have little likelihood of contributing to an overall effect. However, many components could still be included that account for a minimal, and potentially unimportant, contribution to overall toxicity. In this study, the LOI was set at 0.5% of the EC50 on the basis of preliminary analysis indicating that fewer than 20 pesticides would be present in the samples and a level of concern of 0.10 (PTI score or sum of TUs). At this level, it is unlikely that important pesticides were eliminated as components, yet simplification of the system was achieved.

Appropriate setting of the LOI is based on the specific characteristics of each problem. The value should be high enough to adequately remove pesticide concentrations that are unlikely to have toxicological significance in regard to the direct effects on the endpoint in question. However, the value must also be low enough to prevent censoring of components that could contribute significantly to toxicity. Several studies have demonstrated the effects of multiple compounds at low levels (Broderius and Kahl 1985; Altenburger et al. 2000). Thus, datasets that potentially involve very complex mixtures (>20 components) might require lower LOI values. In addition, if pesticides are present that have very shallow concentration–response slopes, lower LOI values would be warranted.

PC-determined mixtures

Determination of mixtures by the PC approach allows for greater reduction in the number of components in specific mixtures. By evaluating each sample individually, components that are unlikely to contribute to toxicity can be eliminated. However, this approach does leave the potential of censoring an important component. This is especially true if greater than additive toxicity occurs among some components. For example, chlorpyrifos has been reported to increase the toxicity of esfenvalerate to fathead minnows at a concentration 40 times lower than its EC50 (Belden and Lydy 2006), and atrazine has been reported to increase the toxicity of several organophosphorus insecticides to aquatic insects at concentrations a hundred times below the no-observable-effect concentrations (Belden and Lydy 2000; Lydy et al.

2004). Although these types of interaction cause a concern for any type of censoring procedure, the concern is greater any time the censoring technique is more liberal in removing components.

Common co-occurrence of pesticides

As can be ascertained by reviewing Tables 5 and 6, a few pesticides tended to co-occur very commonly, even though the combination rarely occurred without the presence of other pesticides. For example, the binary combination of atrazine and metolachor occurred in 80% of samples censored by the LOI approach and 57% of samples by the PC approach. However, this combination infrequently occurs without the presence of other pesticide components (<2%). These frequently co-occurring partial mixture pairs might be of interest to test individually because if unexpected joint toxicity occurs, such as a synergistic response, the effect could be quite important in the pesticide usage landscape.

Uncertainties and limitations

As with any study, a series of limitations should be acknowledged in regard to the current work. First, this study was limited by the quality of the databases used to generate the data. The Pesticide Toxicity and ECOTOX databases provide an invaluable resource of toxicity results. However, numerous investigators at various locations collected the data and they used variable methodologies. Thus, the data were not always comparable to each other. The NAWQA database used to obtain our monitoring data contained high-quality data that were consistently collected and had a high degree of quality control. The database was relatively inclusive of nearly all pesticides that are broadly used within the pesticide landscape that was defined. However, the NAWQA data has 2 inadequacies for the purpose of this analysis. First, the data available for this study were from 1994 to 1997; thus, patterns of specific pesticide use could have changed during that time (e.g., glyphosate use has markedly increased since the study period and cyanazine is no longer used). At present, however, both the acetanilide and triazine classes are still among the most heavily used pesticides. Insecticide use has changed significantly, with the discontinuation of many organophosphate insecticides. As more current data become available, this process should be updated continually to reflect the current contamination patterns. The 2nd inadequacy was that samples were not collected frequently enough to reliably characterize short-term, episodic runoff events. Thus, the more persistent pesticides, such as atrazine, have more influence in the analysis than less persistent and more sporadically applied pesticides, such as the organosphosphate insecticides, even though the latter might have substantial effects with only short-term exposures.

The 2nd limitation was the availability of indicator species. Only a few species have adequate data available to attempt this type of analysis. Because most of this testing was conducted with only a few species, extrapolation to estimate risk for other species could require additional uncertainties to be included in the analyses. To broaden the analysis to include a broader selection of species, PTI values for taxonomic groups, as suggested by Munn and Gilliom (2001), were also evaluated, although not reported. In their approach, PTI scores were based on the EC50s across all data available for taxonomic groups including fish, *Daphnia*, and benthic invertebrates. The results for these taxonomic groups were similar to the bluegill and *D. magna* acute results presented. Currently, PTI approaches for taxonomic groups are not available for primary producers.

The 3rd limitation was the predictive quality of the models used to generate PTI values. Even though the PTI and TQ approaches are ranking systems and not specifically mixture models, comparison of the HTQ value and the PTI score makes an assumption that the toxicity of the pesticide mixtures is best represented by a TU or concentration addition approach. For this study, the contamination patterns found for *P. subcapitata* indicated that most of the PTI scores usually were composed of pesticides within the same class or a related class (acetanilide and triazine herbicides). Thus, concentration addition models were probably the most appropriate (Altenburger et al 2003; Lydy et al. 2004). Previous work by Faust et al. (2001) and Junghans et al. (2003) has demonstrated that the algal toxicity of acetanilide herbicide mixtures and triazine herbicide mixtures were well predicted by concentration addition models. To our knowledge, no published reports detail the joint toxicity of combinations of 2-herbicide groups.

However, for any of the mixtures that have not been tested, it is possible that the approach could underestimate toxicity, especially if toxicokinetic interactions were to occur (Lydy et al. 2004; Belden and Lydy 2006). For example, data obtained for *Daphnia*, which was not presented, indicated that organophosphate insecticides often occurred in combination with atrazine in this pesticide usage landscape. Because interactions between atrazine and organophosphate insecticides have been noted for aquatic invertebrates in which greater toxicity than expected was observed (Belden and Lydy 2000; Lydy et al. 2004), concentration addition models might not be a good assumption.

Even if it is known that toxicity other than concentration addition is likely to occur in a specific mixture, it is unlikely that adequate databases would be available because slopes of the concentration response curves, necessary for response-driven models such as independent action (Altenburger et al. 2003; Lydy et al. 2004), are rarely provided. Thus, PTI scores and similar calculations are likely the only approach currently available with literature-derived data.

CONCLUSIONS

To fully estimate the environmental risk caused by exposure to pesticides, the combined effects of pesticide mixtures must be considered. In this study, consideration of pesticide mixtures in streams draining agricultural watersheds dominated by corn and soybean production resulted in increased risk compared with consideration of individual pesticides. However, PTI scores indicated that increased relative toxicity from mixtures was usually less than a factor of 2. The importance of the magnitude of this increase in relative toxicity depends on the needs of the specific risk assessment. In the current study, the highest individual hazard quotients were frequently greater than 1, especially for *P. subcapitata*. Thus, any increase in hazard should be considered in estimating risk.

The pesticide mixtures within the corn–soybean pesticide landscape tended to be less complex than would have been predicted on the basis of the number of pesticides used within the crop system or on uncensored monitoring data. Single pesticides and simple mixtures tended to dominate PTI values and, thus, the estimated relative toxicity. In addition, most of

the dominant components of the mixtures were within a few pesticide classes. Furthermore, the number of components and the number of mixtures found after using the LOI and PC techniques were much lower than would have been predicted. Thus, although the number of pesticides present within the system might statistically indicate a large variety of potential mixtures, investigators might be able to initially focus on only a few mixtures that are predicted to be the most important. In the corn–soybean pesticide landscape, nearly all important pesticides for algae were herbicides from 2 chemical classes: Acetanilide and triazine herbicides. Future work is needed to determine whether the pesticide contamination patterns in other pesticide usage landscapes have similarly low complexity or whether this is a unique landscape. For example, in more diverse agricultural regions, such as California's Central Valley, USA, the pesticide use patterns are much more complex.

The techniques described in this manuscript provide an initial framework that is useful in assessing the risk of pesticide mixtures. The methodology allows the investigator to estimate the likely effect of not considering mixtures through comparison of PTI and HTQ values. Then, by comparison of the prioritization techniques, the investigator can gain an understanding of the mixtures that are likely to be the most important within the system, allowing further testing and analyses.

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