Complex mixtures of dissolved pesticides show potential aquatic toxicity in a synoptic study of Midwestern U.S. streams

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Appendix A. Study Design and Sampling Sites

A.1. Study Design

The Midwest Stream Quality Assessment (MSQA) was a collaborative study between the U.S. Geological Survey (USGS) National Water Quality Assessment Project (NAWQA) and the U.S. Environmental Protection Agency (USEPA) National Rivers and Streams Assessment Program (NRSA). The MSQA study objectives were, for perennial (year-round flow), wadeable streams in the Midwest, to:

- Determine the status of stream quality—contaminants, nutrients, toxicity, sediment, flow, habitat, and biological communities—across the region;
- Evaluate the relative influence of the measured chemical and physical stressors on biological communities in the streams sampled;
- Evaluate relations between measured stressors and biological communities and the natural and anthropogenic characteristics of the watersheds;
- Develop models and management tools to predict stressors and ecological conditions in wadeable streams across the region.

The MSQA study combined the targeted design commonly used by the USGS NAWQA project (for example, Coles et al. (2012)) and the random stratified design of the USEPA NRSA (Olsen et al., 1999; U.S. Environmental Protection Agency, 2010a). Sampling for MSQA was done at 100 stream sites distributed over an area of about 600,000 square kilometers (km^2), covering parts of 11 States. To meet the site-selection requirements of both the NAWQA and the NRSA programs, 50 of the 100 MSQA sites were selected randomly using NRSA procedures and the remaining 50 sites were selected (targeted) to complete the desired stressor gradients (see Sec. A.2).

Stressors were monitored over a 14-week index period prior to ecological sampling, on the premise that stream ecology is affected by the chemistry, flow condition, and habitat of the streams during a water-quality index period. The objective was to characterize the exposure to chemical stressors for the biological communities during this index period, which was designed to coincide with the high agrochemical use and runoff season (Gilliom et al., 2006). The specific timing (May 7 to August 9) was a compromise, so as to capture runoff after the application of fertilizer and herbicides (typically applied in mid-April to early May), insecticides (which can be applied any time, but generally later in the growing season once infestation has been documented, typically June and July), and fungicides (mostly applied to corn in mid-July to early August). Pesticide application began later than usual in 2013 because cold, wet weather delayed the planting of corn through much of the Midwest (U.S. Department of Agriculture National Agricultural Statistics Service, 2013).

At the end of the index period, ecological surveys were conducted—algae, benthic invertebrates, and fish communities—and physical habitat was assessed at all sites using NRSA protocols (U.S. Environmental Protection Agency, 2013b). MSQA was a multi-stressor study, and the chemical parameters measured in water at MSQA sites included nutrients, suspended sediments, sulfate, chloride, and pesticides. The USEPA NRSA study limits chemical sampling to relatively few parameters and to collection on the day of ecological sampling; thus, much more extensive water-quality sampling was done at MSQA sites over the index period than required for NRSA.

In addition to chemical sampling in water, bed sediments were analyzed for currently used pesticides, halogenated organic compounds, polycyclic aromatic hydrocarbons, and trace metals, and sediment toxicity tests were conducted with amphipods (21-day tests with *Hyalella azteca*) and midge (10-day tests with *Chironomus dilutus*). Passive samplers (POCIS) were deployed in streams for approximately 5 weeks and extracts were analyzed for the same suite of pesticides analyzed in the present study. These

other MSQA study components are not discussed here, but additional findings are published elsewhere (Gellis et al., 2017; Mahler et al., 2016; Moran et al., 2017; Van Metre et al., 2016a; Van Metre et al., 2016b; Waite and Van Metre, 2017). Methods for the comprehensive MSQA study are described in detail in Garrett et al. (in press).

A.2. Site Selection

Both the USEPA NRSA and USGS NAWQA-MSQA approaches were used to achieve the desired mix of sites, spanning land-use gradients and including enough random sites to make statistically valid inferences about the population of streams in the region. The first 50 MSQA sites were obtained from a list of candidate sites generated by the USEPA NRSA Program ("random sites") using a Generalized Random Tessellation Stratified survey design and a sample frame based on stream segments from the National Hydrography Dataset Plus (NHDPlus) (U.S. Environmental Protection Agency, 2010a; U.S. Environmental Protection Agency and U.S. Geological Survey, 2012). The NRSA Program selected candidate sites randomly from stratified groups of stream lines in the NHDPlus dataset, with stratification by State, stream order, and past sampling history. Because the scope of the MSQA study was limited to wadeable streams (which typically have a basin (watershed) size of less than about 2,000 km^2 in the Midwest), NRSA candidate sites with stream order greater than five were excluded from consideration as MSQA candidate sites. The randomly selected candidate sites were evaluated for site suitability, initially using Google Earth™ and secondly with a field reconnaissance visit. If a candidate site was rejected because of lack of appropriate access, safety concerns, lack of permission from the landowner(s), or a likelihood of the stream going dry during the 14-week MSQA sampling period, that site was replaced by the next candidate site in the appropriate State and stream category from the NRSA candidate site list.

The second 50 sites ("targeted sites") were selected to achieve broad coverage of stressor gradients found in the region, based largely on stream and basin characteristics derived from geospatial data sources. Because agriculture is prevalent in the Midwest, the 50 random sites had basins with a wide range in agricultural land use, but none contained more than 8% urban land (as defined in Sec. A.3). Therefore, 12 of the 50 targeted sites were selected to provide a characterization of water quality for streams in urban areas of the Midwest. These urban indicator sites had 18–88% urban land within the basin, and were located in or near seven major cities—Chicago, Illinois; Dayton and Cincinnati, Ohio; Indianapolis, Indiana; Kansas City, Missouri; Milwaukee, Wisconsin; and Omaha, Nebraska. The remaining 38 targeted sites were selected either to represent reference or least disturbed land within the region or to include sites with long-term historical data in order to provide a temporal context for the 2013 study year. Sites with active streamflow gages were prioritized for all 50 targeted sites to provide continuous flow data. Targeted sites were dispersed across the study area to help ensure that the final 100 study sites covered a wide range in other important stream and basin characteristics, such as soil impervious layer, base-flow index, tile drains, and soil type. The final 100 sampling sites are listed in Table A.1.

A.3. Watershed Characteristics

Typically, a national grid for each type of geospatial data was overlaid on the basin boundary defined by the drainage area of the NHDPlus stream network upstream from the site, and the geospatial data were processed by taking the mean of the grid cells or the percentage of cells coded by classifications (such as land use) that overlapped the relevant drainage area for each NHDPlus stream segment in the study area (Nakagaki et al., 2016b). Important site characteristics considered during site selection included urban land within the basin, riparian natural vegetation, and an Agricultural Intensity Index (Aii). The percentage of urban land was defined as the sum of low-, medium-, and high-density developed land (categories 22-24) from the 2011 National Land Cover Database (NLCD) (Homer et al., 2015; U.S.

Geological Survey, 2014a). Riparian natural vegetation consisted of the sum of the following NLCD land cover types (percentages) within a 100-m riparian buffer zone: deciduous, evergreen, and mixed forest; shrub/scrubland; grassland; woody wetland; and emergent herbaceous wetland (categories 41-43, 52, 71, 90, 95). The riparian zone was defined as the boundary defined by NHDPlus stream segment intersecting the candidate site, buffered by 100 meters on each side. The Aii was based on two equally weighted basin characteristics: the percentage of cropland in the basin (i.e., cultivated crops, category 82 from NLCD) and the toxicity-weighted pesticide use (TWU). The TWU was calculated by estimating the agricultural use of each pesticide within the basin (i.e., 2009 county-level high pesticide use estimates from Stone (2013) based on Thelin and Stone (2013)); dividing each pesticide use estimate by the median toxicity concentration of that pesticide towards cladocerans (Nowell et al., 2014), to represent agricultural use normalized by relative toxicity; summing the toxicity-normalized use estimates for all pesticides applied in the basin; and dividing by basin area. Basin land use is provided for the 100 sampled sites in Table A.1, and a larger dataset of basin and riparian characteristics is provided in Nakagaki et al. (2016a).

A.4. Discrete Water Sample Collection

Twelve water samples were collected approximately weekly during 14 weeks, except that a single sample was collected during each of two 2-week periods that included the Memorial Day and July 4th holidays. Discrete water samples were collected and processed using protocols documented in the USGS National Field Manual (U.S. Geological Survey, variously dated), which are designed to ensure representative bias-free samples. For most constituents, multiple incremental samples from across the channel were collected using DH-81, DH-95, or D-95 samplers and immediately composited. When stream velocities, depth, and safety allowed, multiple vertical sampling followed an isokinetic, equal-width increment (EWI) or equal-discharge increment (EDI) approach (U.S. Geological Survey, 2006). Two-person sampling crews used “clean-hands/dirty-hands” roles and pre-cleaned, acid- and methanol-rinsed Teflon sample collection equipment, which included a 14- or 8-L churn for sample compositing.

Subsamples of composited water were withdrawn from the Teflon churn splitter for analyses of various constituents. For pesticide analyses, samples were filtered in the field through a 0.7-µm glass fiber filter into amber glass vials (Sandstrom and Wilde, 2014) and shipped on ice either to the USGS National Water Quality Laboratory, Denver, CO (for analysis of 227 pesticide compounds), or to the USGS Texas Water Science Center laboratory, Austin TX (for analysis of glyphosate).

Appendix B. Chemical Analysis and Quality Control

Pesticide concentration data for environmental samples are available in Nowell et al. (2017).

B.1. Broad-spectrum LC-MS/MS Method

B.1.1. Analytical method

All pesticide compounds except glyphosate were analyzed at the USGS National Water Quality Laboratory (NWQL) using a direct-aqueous injection liquid chromatography tandem mass spectrometric method (LC-MS/MS) that measures 227 pesticide compounds (111 parent pesticides and 116 degradates) with reporting levels in the ng/L range (Sandstrom et al., 2015). The target analyte list (shown in Table B.1) was the result of a pesticide prioritization effort in which high priority was assigned to pesticides with high use, frequent past occurrence in surface or groundwater, or potential toxicity (Norman et al., 2012). Degradates of high priority parent pesticides also were assigned high priority. The method involves direct injection of a 100-microliter (µL) sample of water onto the LC-MS/MS. Samples were analyzed with two injections—one in electrospray ionization (ESI) positive mode and one in ESI negative mode—using dynamic multiple reaction monitoring (MRM) conditions, with two MRM transitions for each analyte. Recoveries for most analytes ranged from 80 to 120% in the water types tested, with relative standard deviations of less than 30%. The reporting levels for most analytes ranged from 1 to 250 ng/L (most below 75 ng/L) for 182 analytes analyzed in the ESI positive mode, and from 2 to 250 ng/L (most below 100 ng/L) for 45 analytes analyzed in the ESI negative mode (Sandstrom et al., 2015).

Table B.1. Pesticide analyte information, analytical reporting levels, and aquatic-life benchmark values. (Excel file)

The MSQA study took place during the first year (2013) that the analytical method was brought into routine operation at the NWQL. The method reporting levels (MRL) discussed in the main paper refer to Interim Reporting Levels (IRL), which were derived on the basis of method performance during method development and prove-out. MSQA data were retrieved from the National Water Information System (NWIS) database on November 21, 2015 (U.S. Geological Survey, 2015c).

Quality control (QC) consisted of surrogates, laboratory blanks, and laboratory reagent spikes (Sec. B.1.2); and field blanks, field matrix spikes, and replicates (Sec. B.1.3).

B.1.2. Laboratory Quality Control

Laboratory quality control at the NWQL consisted of isotopically labeled surrogate compounds added to all samples, plus laboratory reagent spikes and reagent blank samples. Surrogate compounds are not found in the environment (e.g., deuterated and ^{13}C-labeled pesticides), but have similar physical/chemical properties to different groups of targeted analytes, and are added to provide information on method recovery and matrix effects. Twenty-one surrogate compounds were added to all samples prior to analysis, and had mean recoveries ranging from 83% to 123% (Table B.2).

Table B.2. Pesticide surrogate recoveries in environmental samples from the Midwest Stream Quality Assessment. (Excel file)

Reagent blank samples were prepared using reagent-grade blank water, and went through the same preparation and other steps as field samples before analysis. Low-level contamination of some compounds in laboratory reagent blanks was used by the NWQL to set reporting levels for individual analytes, and results are not shown here. The NWQL reports censored values (i.e., non-detections) with a remark code of "<" and a result equal to a reporting level (e.g., <5 ng/L), which indicate that an analyte was not detected at or above that reporting level concentration. A raised reporting level refers to a

censored value where the result value is greater than the IRL (e.g., <25 ng/L for an analyte with an IRL of 5 ng/L). The NWQL reported some MSQA results as censored at a raised reporting level because of interference, loss of instrument sensitivity, sample dilution, partial qualitative identification (i.e., some but not all criteria for identifying the analyte were met), or contamination in a laboratory reagent blank analyzed within the same analytical batch. An analytical batch consists of instrument blanks, calibration standards, third-party check standards, laboratory spikes, laboratory reagent blanks, and environmental samples that are analyzed in a sequence (Sandstrom et al., 2015). The reason for the raised reporting level typically is reported in the VAL_QUAL_CD_TX, or value qualifier code, field of the NWIS database (U.S. Geological Survey, 2015c). For environmental samples associated with one or more contaminated laboratory blank(s), the NWQL applied a value qualifier code of "v" as described in U.S. Geological Survey National Water Quality Laboratory (2011). In summary, a v-code was applied in two situations: (1) If the concentration measured in an environmental sample (Cs) was more than the reporting level but less than three times the highest laboratory blank concentration (Cb) from the same analytical batch, then that environmental sample was censored, v-coded, and reported as less than the concentration measured in that sample (<Cs). (For example, if the blank had a concentration of 10 ng/L, and the environmental sample had a measured concentration of 25 ng/L, that environmental sample would be reported as <25 ng/L to account for potential high bias in the measured concentration and a v-code would be applied.) (2) If Cs was more than three times, but less than ten times, higher than Cb, then the result was reported as a detection (Cs), but with a remark code of "E" to indicate that the reported concentration is estimated, and a v-code was applied. No v-code was applied in two situations: (3) if Cs was more than ten times higher than Cb, in which case the result was reported as Cs without an "E"; and (4) if Cs was less than three times Cb and Cs was also less than the reporting level, in which case the result was reported as less than the reporting level (<IRL).

Altogether, censored data with a raised reporting level constituted about 10% of the total results for the present study. As described in Sec. 2.7 of the paper, results with raised reporting levels were retained in most statistical tests (e.g., generalized Wilcoxon test, Kaplan-Meier test, survival analysis using maximum likelihood estimation), where the procedures used could accommodate multiple censoring levels. In other types of data analysis (e.g., calculation of total detection frequencies), results with raised reporting levels that are greater than the next highest calibration standard above the IRL were dropped from the dataset (i.e., considered to be missing data) because the analysis did not meet the desired laboratory analytical resolution. Calibration standards were 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1,000, 5,000, and 10,000 ng/L for each analyte (Sandstrom and Wilde, 2014). As an example, the IRL for atrazine is 5 ng/L, so the next highest calibration standard is 10 ng/L. When calculating total detection frequencies (shown in Fig. 2, Tables E.1 and E.2), censored results ranging from <5 to <10 would be retained in the dataset and considered to be nondetections, but higher raised reporting levels, such as <25 or <250 would be treated as missing data because the occurrence of atrazine could not be ascertained with an acceptable degree of resolution. This total detection frequency is considered an "upper bound," because some of the dropped results (those with raised reporting levels) may be nondetections. The "lower bound" total frequency was calculated by assuming all censored values with raised reporting levels are nondetections. Table B.1 lists the IRL and the next calibration standard for each pesticide analyte.

Laboratory reagent spikes (LRS) prepared using reagent-grade blank water also provided information on recoveries typical for the analytical method. Analysis of LRS samples showed median recoveries of 94% for ESI positive mode and 98% in ESI negative mode during water years 2012-2013 (Mark Sandstrom, USGS National Water Quality Laboratory, written communication, Data quality of direct aqueous-injection liquid chromatography-tandem mass spectrometry pesticide method during implementation and use by NAWQA Program in 2012 – 2013, May 25, 2016). A few pesticide compounds had median

LRS recoveries of <70% or >130%; these compounds were characterized with an "E" (for estimated) validation quality code because of higher bias or variability in recovery (Table B.1), so all reported concentrations were designated with an "E" remark code (Sandstrom et al., 2015).

Analysis of LRS samples at the NWQL over time indicated consistent data quality during method implementation from May, 2012, to September, 2014, except for the period between May and July, 2013, when LRS recovery showed a pronounced drop for 17 pesticide compounds; this was attributed to degradation of these compounds in the vials of spike mixture used to prepare both LRS and field matrix spikes during this time period (Mark Sandstrom, USGS National Water Quality Laboratory, written communication, Data quality of direct aqueous-injection liquid chromatography-tandem mass spectrometry pesticide method during implementation and use by NAWQA Program in 2012 – 2013, May 25, 2016). As a result, the certification period for spike solutions for this method subsequently was shortened from 1 year to 6 months (U.S. Geological Survey National Water Quality Laboratory, written communication, Rapi-Note 16-10, Matrix spike solutions for NWQL laboratory schedule 2437, May 25, 2016). For the purposes of this study, both LRS and field spikes prepared with the degraded spike solution were considered to have been compromised and therefore unsuitable for use in assessing method performance.

B.1.3. Field Quality Control

The field QC samples collected for MSQA—92 matrix spikes, 42 blanks, and 50 pairs of replicate samples (duplicates)—corresponded to 15% of the environmental samples.

Of the 42 field blanks, 12 blanks had one or more compounds detected, and 11 total compounds were detected in one or more blanks (Table B.3). No data adjustments were made for 8 of the 11 compounds. Of these, six compounds were detected in only a single blank and the concentration detected was below the reporting level. Diuron was detected in two blanks, but again the concentrations were below the reporting level. Azoxystrobin was detected in a single blank at a concentration twice the reporting level, but no azoxystrobin was detected in the corresponding environmental sample. Data adjustments were made for the other three compounds that were detected in field blanks. Metolachlor was detected in 19% of field blanks, but at low concentrations (0.7-4.6 ng/L), below the IRL (9 ng/L) and much lower than the concentrations detected in the corresponding environmental samples collected at the same site on the same date (7- to 940-fold higher; Table B.4). Metolachlor also was detected at similar concentrations in 10% of laboratory blanks analyzed in the same sets with the MSQA samples, suggesting the possibility of occasional, low-level incidental contamination. To account for the possibility that low-level incidental contamination may have occurred in environmental samples, metolachlor concentrations in all environmental samples were censored at the IRL (9 ng/L). Therefore, the "total" detection frequencies of metolachlor shown in Fig. 2 (and listed as upper-bound detection frequencies in Tables E.1 and E.2) represent detections > 9 ng/L, and may underestimate its occurrence in MSQA streams. The most sensitive benchmark for metolachlor (Chronic-Invertebrate benchmark, 1,000 ng/L) was over 200-times above the highest field blank detection, so any incidental contamination that may have occurred in environmental samples would not have contributed to benchmark exceedances. Two compounds, 2,4-D and triclopyr, were detected in a single blank sample (the same blank) at high concentrations—higher than the concentrations in the corresponding environmental sample (Table B.3). It is possible that equipment was accidentally contaminated by contact with treated vegetation at the sampling site, as both of these herbicides are used in nonagricultural/roadside weed control. Data for the corresponding environmental samples were dropped from analysis because incidental contamination of field equipment at the site could not be ruled out.

Table B.3. Detections of pesticide compounds in field blanks from the Midwest Stream Quality Assessment. (Excel file)

Table B.4. For MSQA field blanks with detectable metolachlor, comparison of concentrations in field blanks with the corresponding environmental samples. (Excel file)

In the 50 field replicate pairs, 86 compounds were detected in both members of at least one pair; the mean relative percent difference (RPD) was <20% for 52 compounds (60% of all compounds) and <40% for 82 compounds (95% of all compounds) (Table B.5). Two compounds had high variability, but both were based on a single replicate pair with detections: chlorpyrifos (RPD of 62%) and ethoprop (49%). Another measure of variability is the percentage of inconsistent detections (detection in one sample paired with a nondetection that has a lower reporting level than the detected concentration). Only 45 analyte/replicate combinations (0.8%) were inconsistent detections, and most of these were at concentrations near (within a factor of two) the reporting level, where variability tends to be highest. Only 4 analyte/replicate combinations (0.07%), therefore, were inconsistent at concentrations more than twice the IRL.

Table B.5. Analysis of variability in paired field replicate samples from the Midwest Stream Quality Assessment. (Excel file)

The 17 analytes observed to have degraded in LRS during May-August of 2013 (Sec. B.1.2) also had very low recoveries in MSQA field spikes, as did some additional analytes (Shoda et al., in press). Because all MSQA field matrix spikes were prepared during May-August, 2013, using a faulty spike solution (Sec. B.1.2), the MSQA field spikes were considered to underestimate method performance and are not reported here. Instead, method performance was assessed using 289 field spike samples prepared during the time period from December 3, 2012, through September 10, 2015, using water samples from 207 streams and rivers sampled nationwide by other NAWQA studies (Table B.6). These field spikes were prepared using the same water sampling and spiking procedures, and analyzed using the same pesticide analytical method used in the MSQA study. These NAWQA field spikes also bracket the MSQA study in terms of sampling date, so they provide supplemental information on method performance in surface water field matrices. Data processing steps are described in Shoda et al. (in press). Based on the NAWQA field spikes, median recoveries were in the 70-130% range for 200 of 221 analytes (90%), with an additional 14 analytes in the 50-70% range. Three analytes (degradates diketonitrile isoxaflutole, OEAT, and CAAT) had median recoveries >130% in field spikes, indicating that measured concentrations may be biased high. Four analytes had <50% recovery, of which only one (1H-1,2,4-triazole) was detected in more than 1% of MSQA samples; measured concentrations of these 4 analytes may be biased low (Table B.6). For 1H-1,2,4-triazole (a degradate of the fungicide propiconazole), detections of this compound are valid, but the detection frequency is difficult to calculate because a high percentage (83%) of censored data had a raised reporting level; depending on whether censored results with reporting levels raised above the next calibration standard were counted as nondetections or were dropped as missing data, the detection frequency for this degradate could be anywhere from 5% to about 30% of samples overall (Tables E.1 and E.2). The analytes with median field spike recoveries <70% or >130% are so designated in Tables E.1 and E.2, which report summary statistics for pesticide compounds at Agricultural-gradient and Urban sites, respectively. Variability in field spike recovery is represented by the F-pseudosigma, which is the interquartile range divided by 1.349. The F-pseudosigma is a non-parametric statistical measure of variance, similar to the standard deviation, that is used to reduce the influence of statistical outliers relative to the more commonly used parametric version. It is useful for characterizing symmetric data containing outliers at both ends (as in the present dataset of field spike recoveries), providing a more resistant measure of spread than does the standard

deviation (Helsel and Hirsch, 2002). Fourteen compounds have F-pseudosigma values >30%, indicating that these compounds have higher variability than the remaining compounds; nine of the 14 were flagged as having median recoveries of >130% or <70%. Because the field spikes prepared from the MSQA water samples were faulty, matrix effects in MSQA streams could not be evaluated in the present study.

Table B.6. Recovery of pesticide analytes in field spikes collected in surface water at National Water Quality Assessment (NAWQA) sites (Dec 2012-Sept 2015). (Excel file)

B.2. Glyphosate Analysis by ELISA

B.2.1. Analytical Method

Glyphosate was analyzed in a subsample (split) of all environmental water samples using Enzyme-Linked Immunosorbent Assay (ELISA) at the USGS Texas Water Science Center laboratory. Samples were analyzed using 92-well microtiter plate kits (including all reagents) and analytical protocols provided by Abraxis (Warminster PA) (http://www.abraxiskits.com/products/pesticides/). Details are provided in Mahler et al. (2016). Briefly, a 50 µL sample was derivatized with reagent and buffer, and the derivatized sample and an antibody specific for glyphosate were pipetted into 92 microtiter wells pre-coated by the manufacturer with goat anti-rabbit antibody, then incubated at room temperature. An enzyme conjugate was added, causing a competitive reaction with glyphosate in the sample for the antibody binding sites on the microtiter well. The wells were washed and a solution containing an enzyme substrate and a chromogen was added. After incubation, a dilute acid solution was added to stop the reaction. The color developed as a result of the reaction was inversely proportional to the glyphosate concentration in the sample. The absorbance associated with each well in the microtiter plate was analyzed photometrically, and the concentration was calculated on the basis of a standard curve. Six standards, a control, and variable numbers of replicates, blanks, and spiked environmental samples were included in each 92-well microtiter plate analysis. Per the manufacturer, the method is ≤0.1% cross-reactive with other compounds.

Although results are not presented in the present study, selected weekly samples collected at 27 sites (160 samples) also were analyzed for glyphosate by LC-MS/MS at the USGS Organic Geochemistry Research Laboratory in Lawrence, KS (Meyer et al., 2009). The methods, results, and a comparison between the ELISA and LC-MS/MS methods are published elsewhere (Mahler et al., 2016).

B.2.2. Laboratory Quality Control

In 25 laboratory blanks, the median glyphosate concentration measured was 70 ng/L and the interquartile range was 0–120 ng/L. On the basis of the blank results, the method reporting level MRL was set at 200 ng/L.

A control was provided with each glyphosate ELISA kit by the manufacturer, and 2–8 controls were run in each 92-well plate. For the 14 batches run with two controls, the median relative percent difference (RPD) in controls was 8% with an interquartile range of 4–13%. The 15 runs with >2 controls had a median RPD of 13% and an interquartile range of 9–17%.

Laboratory spiked samples were run in each plate. The 136 spikes had a median recovery of 115% and an interquartile range of 89–119%

B.2.3. Field Quality Control

All 58 field blanks analyzed by ELISA had a glyphosate concentration less than the reporting level of 200 ng/L. The 47 replicate sample pairs collected had a median RPD of 11% and an interquartile RPD range of 0–36%. Details are provided in Mahler et al. (2016).

B.3. Pesticide Use

For each of the frequently detected pesticides shown in Fig. 2 of the main paper, Table B.7 provides quantitative estimates of agricultural use within the entire MSQA study area in 2013, along with a list of major crops accounting for 5% or more of agricultural use within the 11 MSQA States. These data are from Baker and Stone (2014). Occasionally, there was negligible agricultural use within the MSQA study area, but reported use within the 11 States (e.g., for diazinon and fipronil). Although quantitative data are not available for nonagricultural pesticide use, Table B.7 lists some major nonagricultural applications for each pesticide, typically obtained from USEPA registration documents (cited in Table B.7).

Table B.7. Pesticide use information for the most frequently detected pesticides in streams from the Midwest Stream Quality Assessment (MSQA), as listed in Figure 2 of the paper. (Excel file)

Appendix C. Potential Aquatic-Toxicity Screening Methods

C.1. Supplemental Aquatic-Life Benchmarks

Office of Pesticide Program (OPP) aquatic-life benchmark values were obtained from U.S. Environmental Protection Agency (2017a) and used as toxicity thresholds as described in the main body of the paper. Benchmarks for fenamiphos degradates were not available from OPP, so were computed from toxicity data in USEPA's Fenamiphos Environmental Risk Assessment (U.S. Environmental Protection Agency, 1999) using the same procedures that OPP uses to derive benchmarks. For atrazine, additional thresholds from USEPA's refined risk assessment for atrazine (Farruggia et al., 2016) for fish reproduction (5,000 ng/L) and aquatic plant community primary productivity, structure, and function (3,400 ng/L) were applied as chronic atrazine benchmarks for fish and plants, respectively. Both were compared with 60-day average concentrations (as per Farruggia et al., 2016), so were considered to be chronic thresholds. OPP recently (Feb. 3, 2017) adopted the fish reproduction threshold as a Chronic-Fish benchmark (U.S. Environmental Protection Agency, 2017b).

The lower incidence of benchmark exceedances observed for Vascular Plants (at 11% of sites) compared to Nonvascular Plants (74% of sites) is due in part to the lack of a quantitative atrazine benchmark for Vascular Plants in the present analysis. The former OPP Acute Vascular Plant (Acute-VP) benchmark for atrazine (0.001 µg/L) was omitted from benchmark quotient calculations in this study because U.S. Environmental Protection Agency (2013a) stated that the toxicity value underlying the benchmark is the "most sensitive single species data for aquatic vascular plants…, [which] will be used for risk characterization purposes only and will not be used for quantitative purposes such as use in risk quotient calculation." OPP recently (Feb. 3, 2017) published a new atrazine Acute Vascular Plant benchmark of 4.6 ng/L (U.S. Environmental Protection Agency, 2017b). When this benchmark was applied to MSQA stream data, the percentage of sites exceeding one or more Acute Vascular Plant benchmarks increased from 11 to 36% of sites—still considerably lower than the 74% of sites with exceedances observed for the Acute Nonvascular Plant benchmark.

C.2. Limitations of Screening Tools

OPP aquatic-life benchmarks are typically based on the most sensitive toxicity test of acceptable or supplemental studies, as evaluated by USEPA as part of pesticide registration. Similarly, toxicity concentrations included in the Pesticide Toxicity Index (PTI) are based on either 5th percentile or (if insufficient data are available) minimum toxicity values available for appropriate test species, endpoints, and durations. This definition corresponds to the Sensitive PTI from Nowell et al. (2014). However, considerable variability occurs in toxicity values for a given pesticide and test organism, depending on factors such as age and condition of the test organism, test conditions, etc. (Nowell et al., 2014). Therefore, uncertainty in toxicity values is high for pesticides with relatively few toxicity tests available.

Both aquatic-life benchmarks and the PTI are limited to pesticides measured in the water column, so hydrophobic pesticides (e.g., pyrethroids and fipronil) may be under-represented in terms of potential toxicity, especially to benthic organisms.

The PTI assumes that toxicity of pesticide mixtures is additive; this assumption tends to overestimate toxicity for mixtures of pesticides with dissimilar modes of action by up to a factor of 2–3 (Belden et al., 2007; Deneer, 2000; Faust et al., 2003; Warne, 2003). However, when a single compound dominates the PTI, as occurred for most samples in the present study (Sec. F.2), the results are similar to using the TUmax (i.e., toxic unit for the pesticide that makes the single largest contribution to the PTI) and the effects of the additivity assumption are minimal.

The PTI is based on short-term exposure and acute toxicity (typically lethality) and does not represent sublethal effects, especially under chronic exposure conditions. In the present study, the use of a PTI threshold of 0.1 to estimate potential chronic toxicity effectively applies an acute-to-chronic ratio (ACR) of 10 to acute toxicity levels (i.e., LC50s that were used to calculate the PTI). This ACR value of 10 falls between the median and 80th percentile of invertebrate ACR values (TenBrook et al., 2010), and is designed to reflect a concentration above which chronic effects are likely. Because an ACR of 10 is not an extremely conservative (protective) choice, chronic effects on aquatic organisms may occur below this threshold.

Degradate toxicity may be greater than, comparable to, or less than toxicity of the parent compound (Sinclair and Boxall, 2003). Aquatic life benchmarks were available for relatively few of the pesticide degradates analyzed in this study, ranging from 3% of degradates (for Chronic-Fish benchmarks) to 18% of degradates (for Acute-Fish and Acute-Invertebrate (Acute-Invert) benchmarks). As a result, many degradates were not counted in potential toxicity estimates.

Results from OPP benchmark comparisons and PTI screening approaches are not identical because (1) these screening tools are based on overlapping, but not identical, datasets of toxicity values, and (2) methods for deriving the PTI and its thresholds (Nowell et al., 2014) differ from those to derive benchmarks (U.S. Environmental Protection Agency, 2017c). For example, OPP Acute-Invert benchmarks are determined to be one-half of the most sensitive short-term LC50 for all invertebrate taxa tested, whereas the PTI toxicity concentrations for cladocerans and benthic invertebrates are set equal to the 5th percentile of short-term LC50s for these two subsets of invertebrate taxa. (For cladocerans, short-term EC50s for immobilization are used, in addition to LC50s, in computing both OPP benchmarks and PTI toxicity concentrations.) OPP Chronic-Invertebrate (Chronic-Invert) benchmarks are typically set equal to the lowest No-Observed-Adverse-Effect-Concentration (NOAEC) from life-cycle tests with all invertebrate taxa, whereas the chronic PTI thresholds are computed separately for cladoceran and benthic invertebrates and correspond to 1/10th of the 5th percentile (or the minimum value, if there are insufficient data to calculate a 5th percentile) of acute LC50s for appropriate taxa. For example, acute and chronic excursions for imidacloprid occur more often based on comparisons with the Benthic invertebrate-PTI than with either the Cladoceran-PTI or OPP benchmarks because the Benthic invertebrate-PTI toxicity concentration (0.65 µg/L) is based on a more sensitive species (96-hr LC50 for the mayfly *Epeorus longimanus*, from Nowell et al. (2014)), than either the Cladoceran-PTI toxicity concentration (832 µg/L, based on 48-hr EC50 for cladocerans, from Nowell et al. (2014)) or the OPP Acute-Invert benchmark (34.5 µg/L, based on an acute EC50 for midge from U.S. Environmental Protection Agency (2008b)).

To calculate acute invertebrate threshold exceedance frequencies for a pesticide in the present study, instantaneous sample concentrations were compared to all three acute thresholds—OPP's Acute-Invert benchmark, or a PTI-TU of 1 for either cladocerans or benthic invertebrates—and samples with any one or more of the three thresholds exceeded were counted as having an acute exceedance. In the present study, chronic threshold exceedances for a pesticide were determined for each site, rather than for individual samples. Specifically, the maximum time-averaged (21-day) concentration of a pesticide at each site was compared to all three chronic thresholds—OPP's Chronic-Invert benchmark, or a PTI-TU of 0.1 for either cladocerans or benthic invertebrates—and any site exceeding one or more of these three thresholds was determined to have a chronic exceedance for that pesticide.

Appendix D. Benthic Macroinvertebrate Sample Collection and Processing Methods

Benthic macroinvertebrate communities were sampled once at 99 of 100 sites during July 22–August 9, 2013, at the end of the water sampling period. (Persistent high flows at one site made sampling using wading methods impossible.) Samples were collected along 11 equally-spaced transects within the stream reach following protocols from USEPA's NRSA Program (U.S. Environmental Protection Agency, 2013b). Assessments were performed by multiple state agencies, USGS Water Science Centers, USEPA personnel and contractors, with all team leaders having attended training on NRSA protocols. The stream reach was defined as 40 times the baseflow wetted channel width, from a minimum of 150 m to a maximum of 4 km. Invertebrate samples were collected at the point 1 m downstream of each transect, at either 25, 50, or 75% of the channel width (Left, Center, Right), successively moving downstream.

A D-frame net with 500 µm mesh openings was used. Organisms were preserved in 95-percent ethanol and shipped to the USGS NWQL Biological Unit, in Denver, CO, for identification and enumeration using methods by Moulton et al. (2000). During taxonomic data processing, the following decision rules were followed: terrestrial adults were deleted, different life stages for a common taxonomic endpoint were combined, and ambiguous taxonomic 'parents' were distributed proportionately among the 'children' so that no distinct taxa endpoints were deleted. Quality Assurance was verified in both the sorting and taxonomic assignment steps by a second person on 10% of the organisms. The Invertebrate Data Analysis System (IDAS) software (Cuffney, 2003) was used to resolve taxonomic issues, remove ambiguous taxa (Cuffney et al., 2007), and generate invertebrate metrics. The raw data of species taxonomy and enumeration are available in the USGS BioData Database (U.S. Geological Survey, 2015a). Four commonly used metrics of the benthic invertebrate community (Moran et al., 2017; Waite and Van Metre, 2017) were used in the present study: Ephemeroptera, Plecoptera, and Trichoptera (EPT, which consists of mayflies, stoneflies, and caddisflies) richness; EPT abundance; Ephemeroptera abundance as a percentage of sample total; and a macroinvertebrate multimetric index (MMI) that is based on NRSA methods and includes six individual invertebrate metrics (Stoddard et al., 2008; U.S. Environmental Protection Agency, 2016).

Appendix E. Pesticide Occurrence in Relation to Land Use: Supplemental Results

Detection frequencies and summary statistics of pesticide compounds in the Midwest Stream Quality Assessment (MSQA) study during May 7-August 9, 2013, are provided in Tables E.1 and E.2 for Agricultural-gradient and Urban sites, respectively. For frequently detected pesticides, Table E.3 provides generalized Wilcoxon test results comparing concentration distributions at Agricultural-gradient and Urban sites. Table E.4 lists the results of Tobit regression models for the maximum concentration of these pesticides (by site) in relation to agricultural pesticide use intensity and land use explanatory variables.

Table E.1. Detection frequencies and summary statistics for pesticide compounds in Agricultural-gradient streams from the Midwest Stream Quality Assessment (MSQA). (Excel file)

Table E.2. Detection frequencies and summary statistics for pesticide compounds in Urban streams Midwest Stream Quality Assessment (MSQA). (Excel file)

Table E.3. Summary of generalized Wilcoxon test results comparing concentration distributions of selected pesticides in Agricultural-gradient and Urban streams. (Excel file)

Table E.4. Summary of Tobit regression results for selected pesticides, in which maximum pesticide concentration (log-transformed) was regressed with agricultural pesticide-use intensity and land use in the basin. (Excel file)

The concentration distributions were significantly different by land use for most of the most commonly detected pesticides in each use group—azoxystrobin being the sole exception (Fig. E.1). Concentration distributions were higher at agricultural-gradient sites for acetochlor, atrazine and metolachlor, and at urban sites for 2,4-D, glyphosate, carbendazim, fipronil, and imidacloprid. The temporal distributions of selected pesticides—selected because of high detection frequency or potential toxicity to aquatic life, or both—are shown in Fig. E.2. Fig. E.3 shows the temporal changes in the ratio of CIAT/atrazine concentration ratio (also called the deethylatrazine/atrazine ratio, or DAR), during the study period. The DAR remained <1 in the majority of samples, indicating that atrazine concentrations were generally higher than concentrations of its degradate CIAT (deethylatrazine). The DAR increased during the second half of the study period, reflecting decreasing atrazine inputs and increasing atrazine degradation during July-August, consistent with the timing of an earlier spring flush (Thurman and Fallon, 1996).

Propazine was detected frequently in agricultural stream samples, despite its negligible use in agriculture in the study area in 2013. A close relation between propazine and atrazine concentrations within the same sample was observed, with a propazine/atrazine mass ratio (slope) of 1%. This suggests that the frequent occurrence of propazine in agricultural MSQA streams arises from its presence as an impurity in atrazine formulations applied in the MSQA study area (Fig. E.4.A). Similar results, including a slope of 1%, were observed in stream samples collected nationwide in 2012 (Martin et al., in press), but at much lower concentrations (Fig. E.4.B) than in the Midwest, where atrazine use on corn is high (Table B.7). Organophosphate (OP) insecticide occurrence was sporadic, and individual OP compounds had different temporal occurrence patterns from one another and by land use (Fig. E.5).

Fig. E.1. Concentration distributions of selected pesticides at Agricultural-gradient and Urban sites: the herbicides (A) Acetochlor, (B) Atrazine, (C) Metolachlor, (D) 2,4-D, (E) Glyphosate; the fungicides (F) Azoxystrobin, (G) Carbendazim; and the insecticides (H) Imidacloprid, (I) Fipronil. Red letters above boxplots, if different, indicate that distributions are not identical, as determined using the generalized Wilcoxon test for censored data (p<0.05).

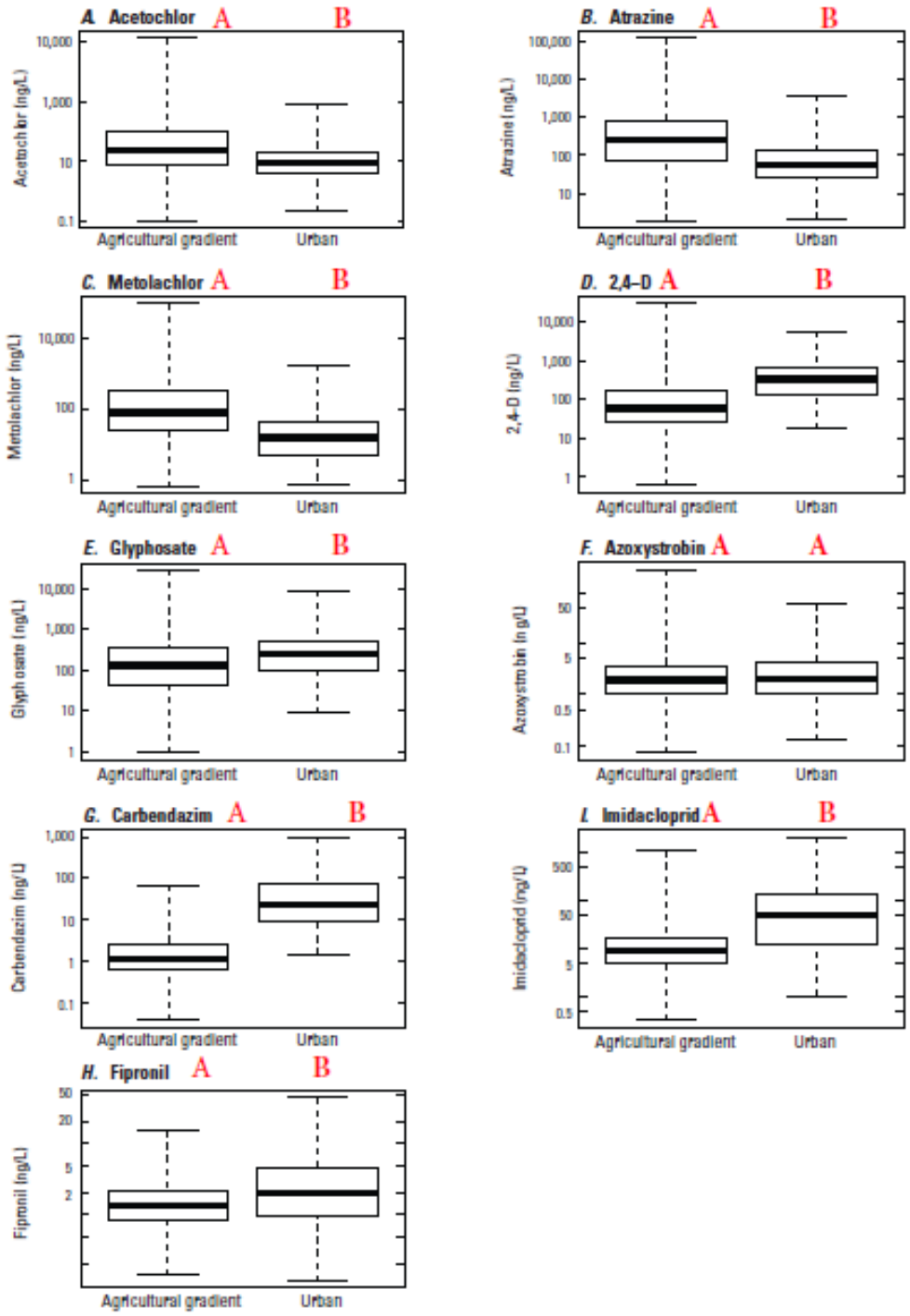


Fig. E.2. Concentrations distributions of selected pesticides, by land use: the herbicides Acetochlor at (A) Agricultural-gradient sites and (B) Urban sites; Atrazine at (C) Agricultural-gradient sites and (D) Urban sites; Metolachlor at (E) Agricultural-gradient sites and (F) Urban sites; 2,4-D at (G) Agricultural-gradient sites and (H) Urban sites; Glyphosate at (I) Agricultural-gradient sites and (J) Urban sites; the fungicides Azoxystrobin at (K) Agricultural-gradient sites and (L) Urban sites; Carbendazim at (M) Agricultural-gradient sites and (N) Urban sites; and the insecticides Imidacloprid at (O) Agricultural-gradient sites and (P) Urban sites; and Fipronil at (Q) Agricultural-gradient sites and (R) Urban sites. Samples with raised reporting levels are included. Mid-date, middle date in sampling week (or in two-week period).

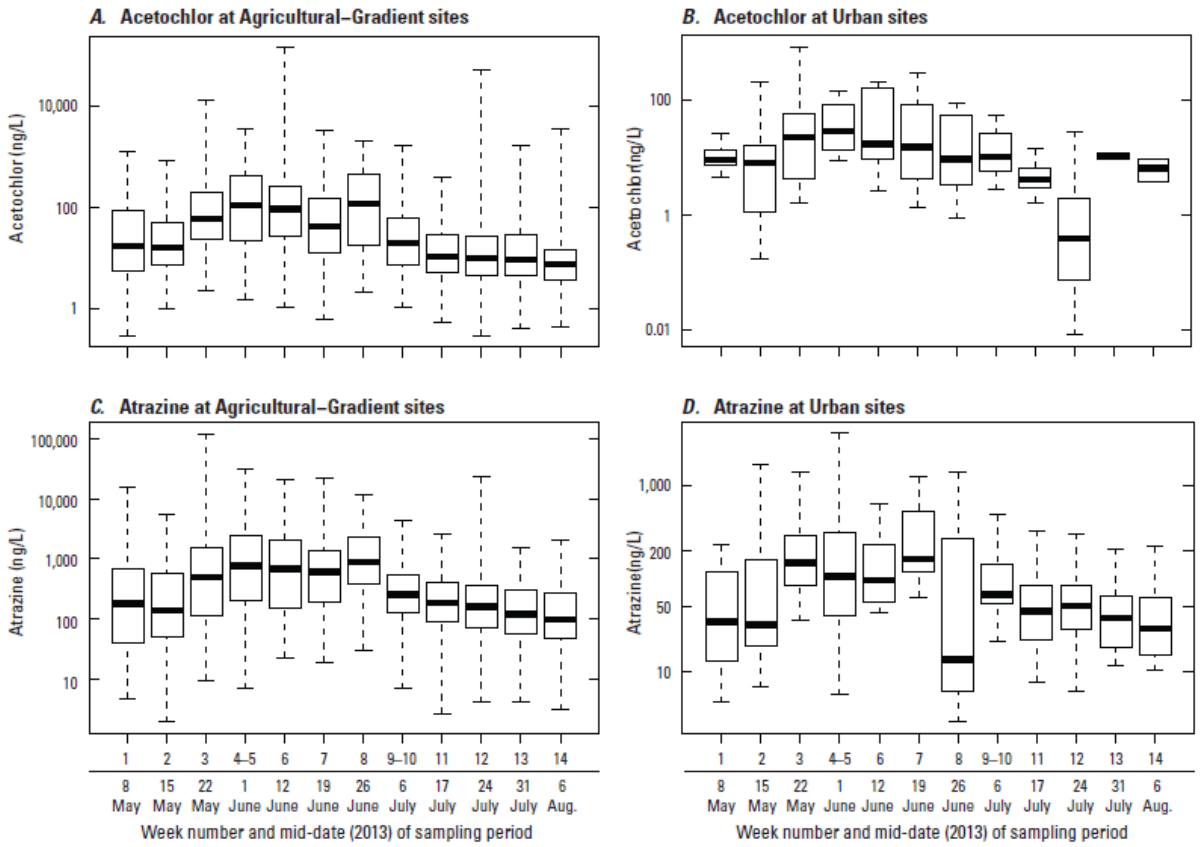


Fig. E.2, Herbicides, cont.

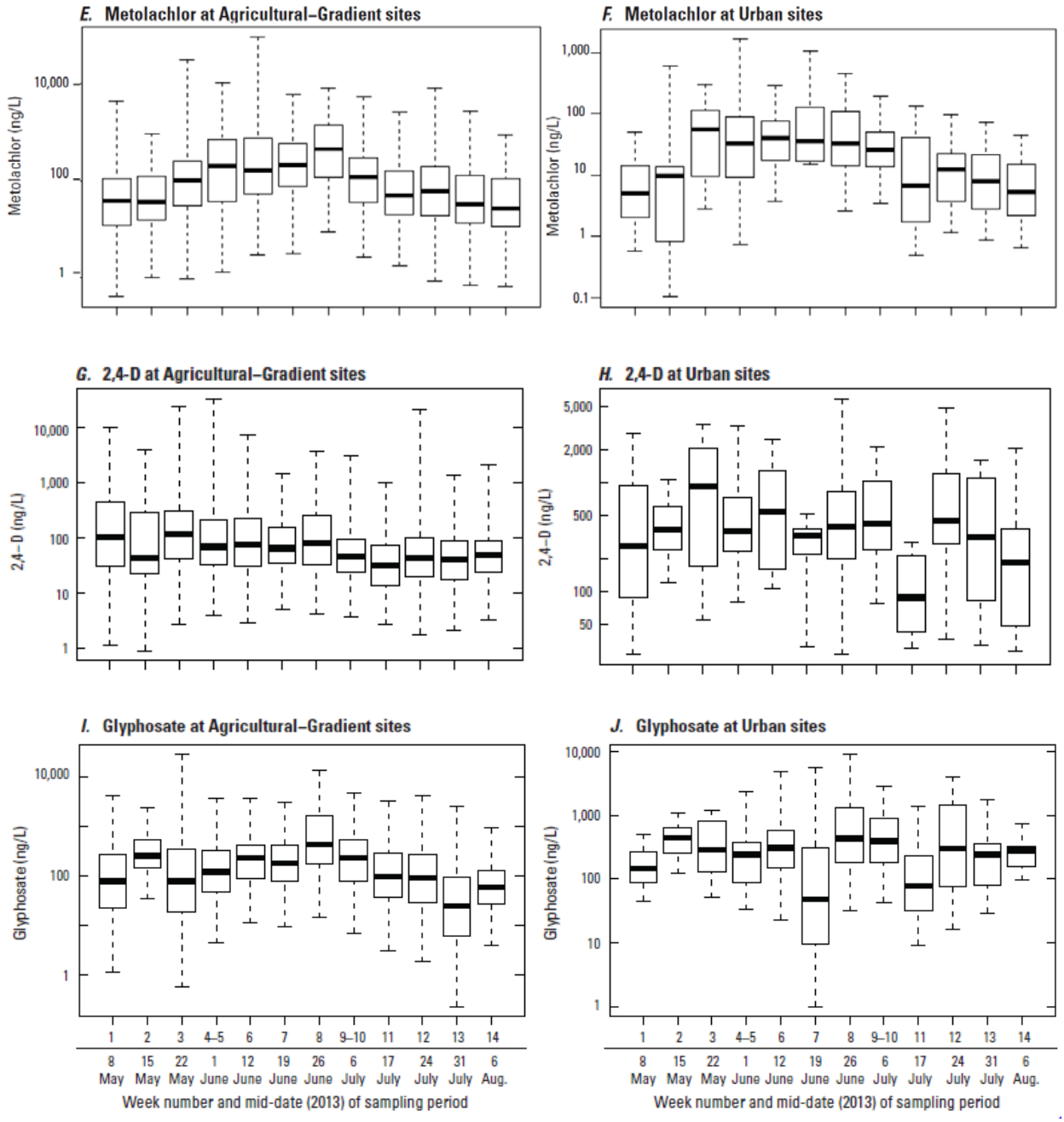


Fig. E.2, cont., Fungicides

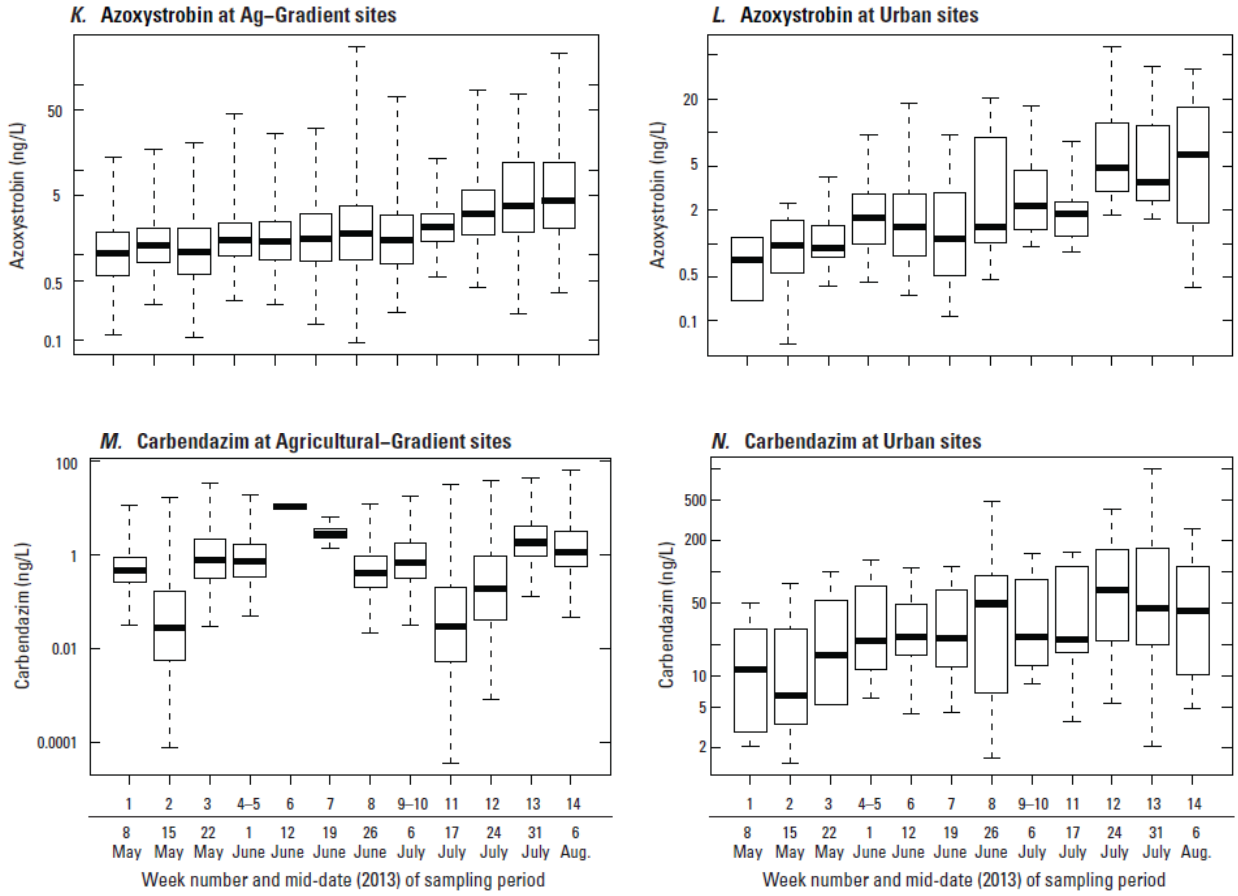


Fig. E.2, cont., Insecticides

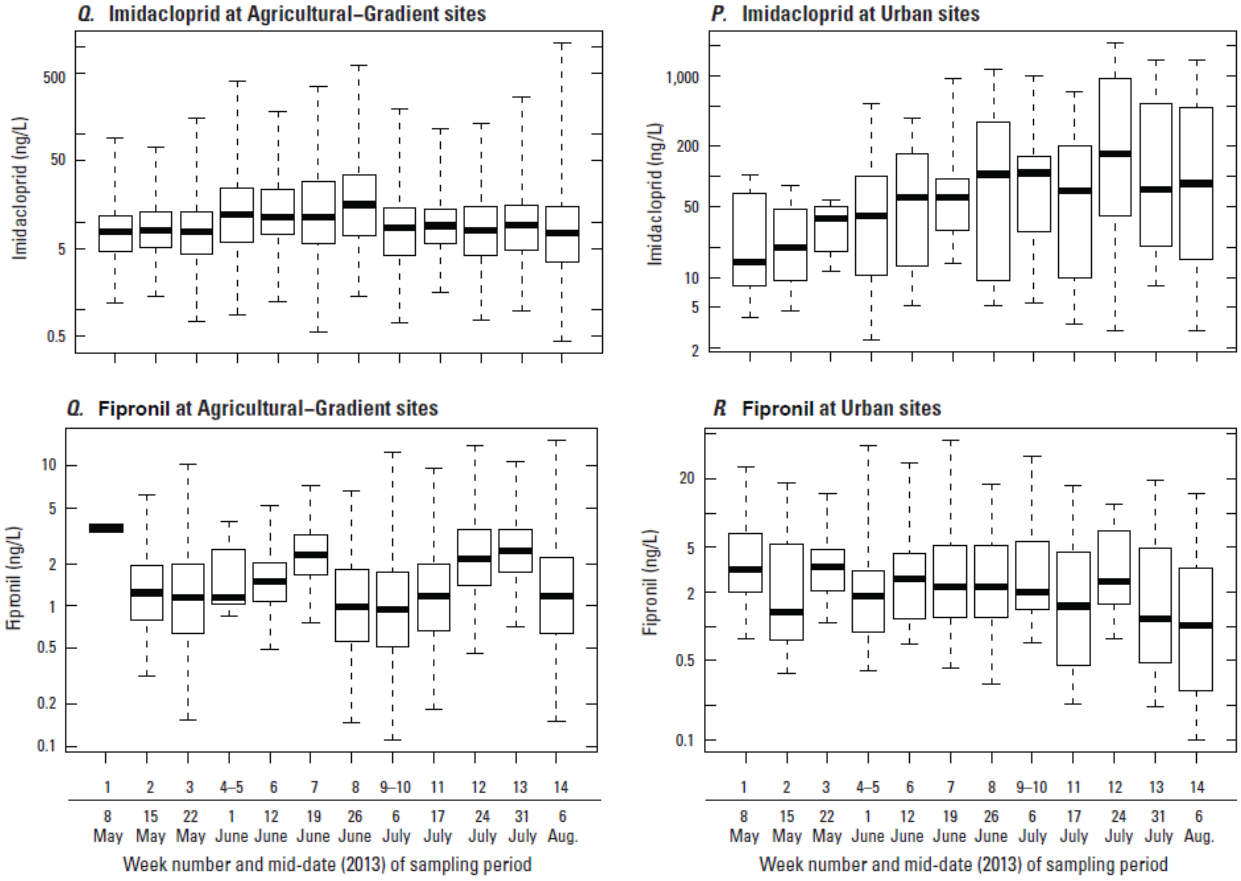


Fig. E.3. Temporal distribution of CIAT/Atrazine concentration ratio (also called Deethylatrazine/atrazine ratio, or DAR) at (A) Agricultural-gradient sites and (B) Urban sites. Mid-date, middle date in sampling week (or in two-week period).

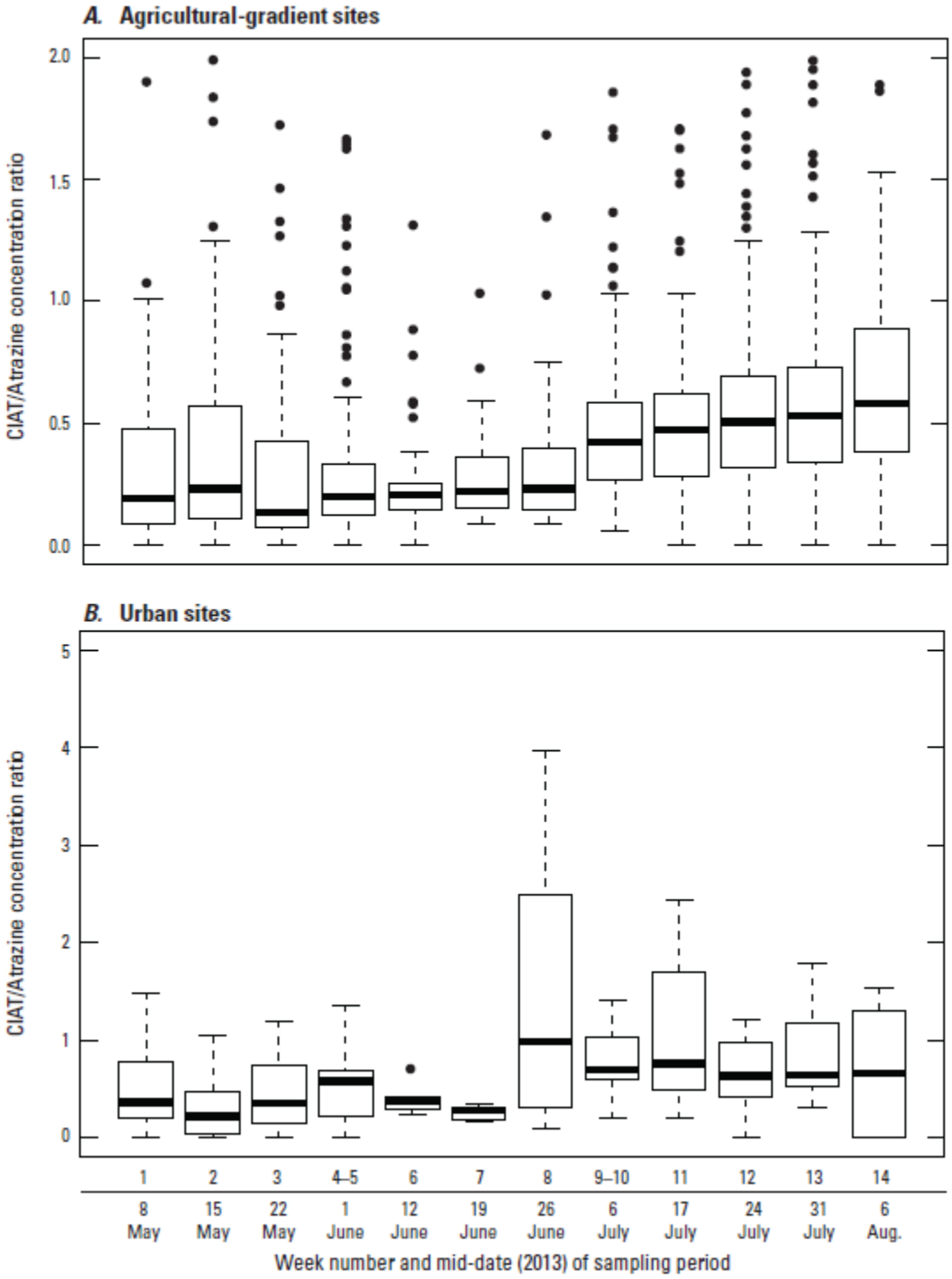


Fig. E.4. The relation between atrazine and propazine concentrations is linear, with a slope of 1% in both (A) Midwest Stream Quality Assessment (MSQA) streams in 2013 and (B) streams sampled nationwide by the National Water Quality Assessment (NAWQA) Project in 2012. The 2012 data are from Martin et al. (in press). Only those samples with propazine detected are shown. One high data point (120,000 ng/L atrazine) is not shown for MSQA streams, but its inclusion did not change the slope of the relation.

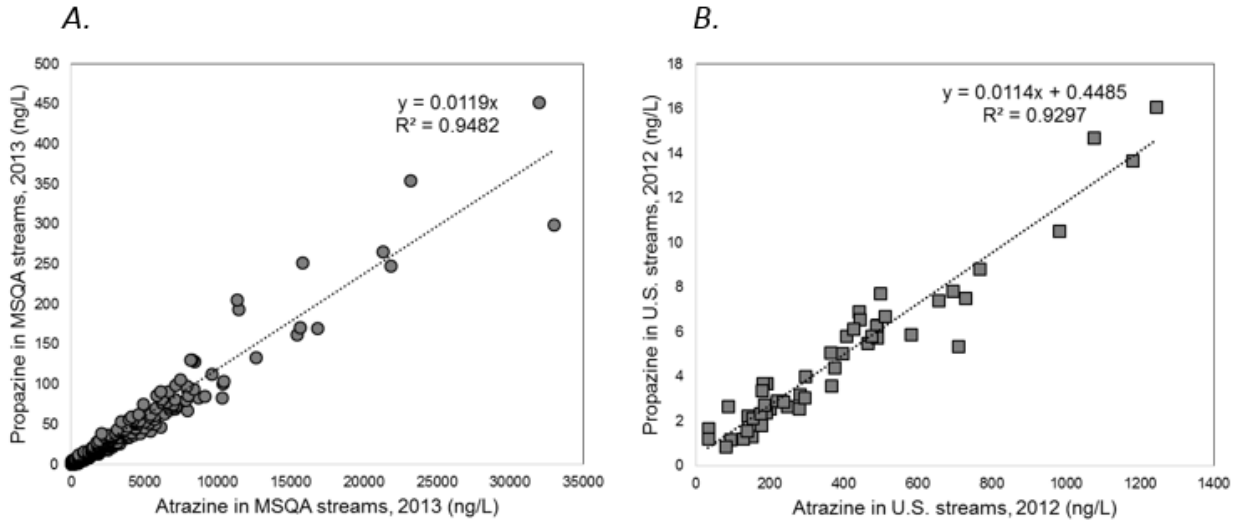


Fig. E.5. The maximum concentrations of individual organophosphate insecticide compounds at (A) Agricultural-gradient sites and (B) Urban sites, by sampling week. Mid-date, middle date in sampling week (or in two-week period). MRL, method reporting level.

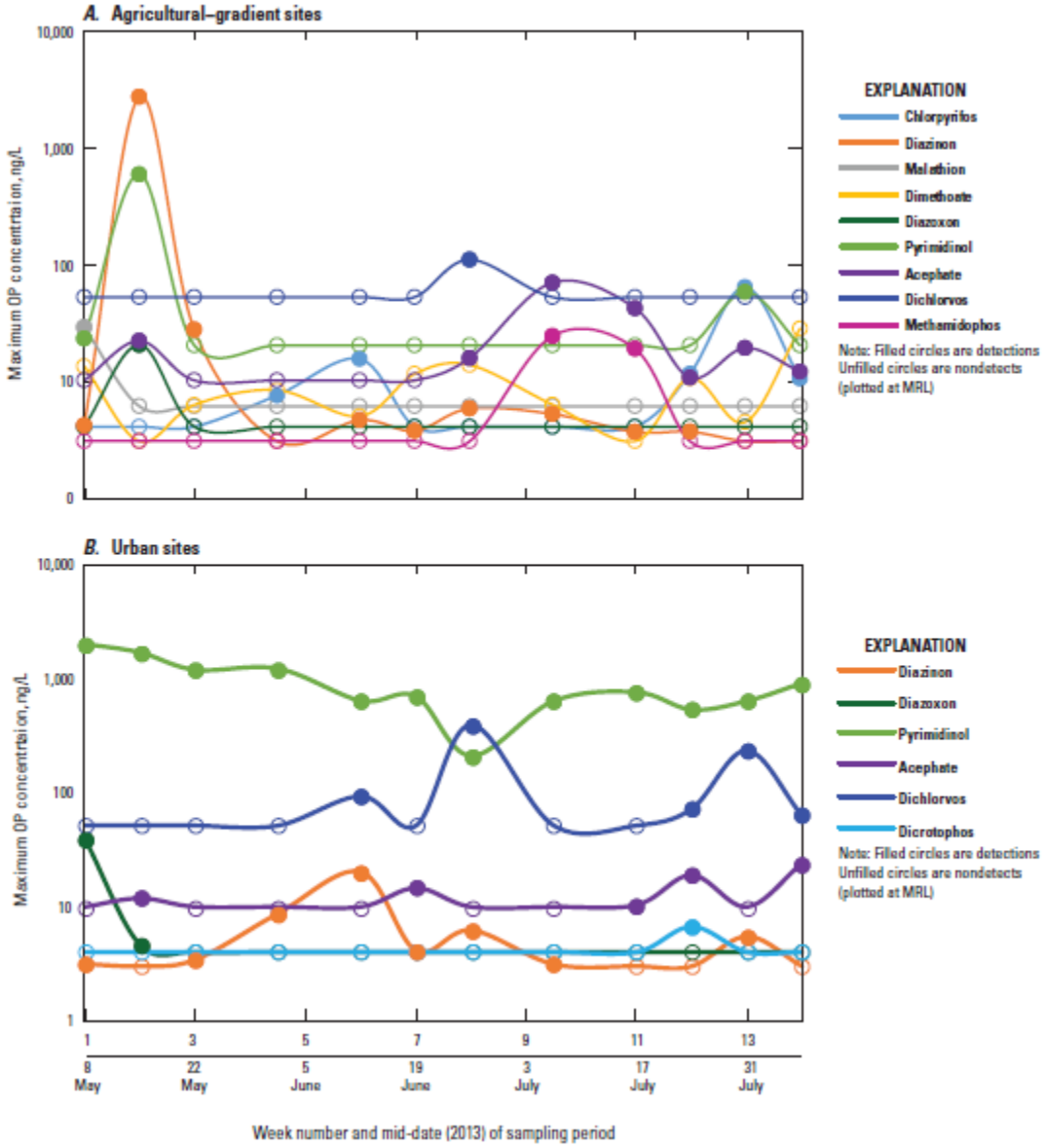
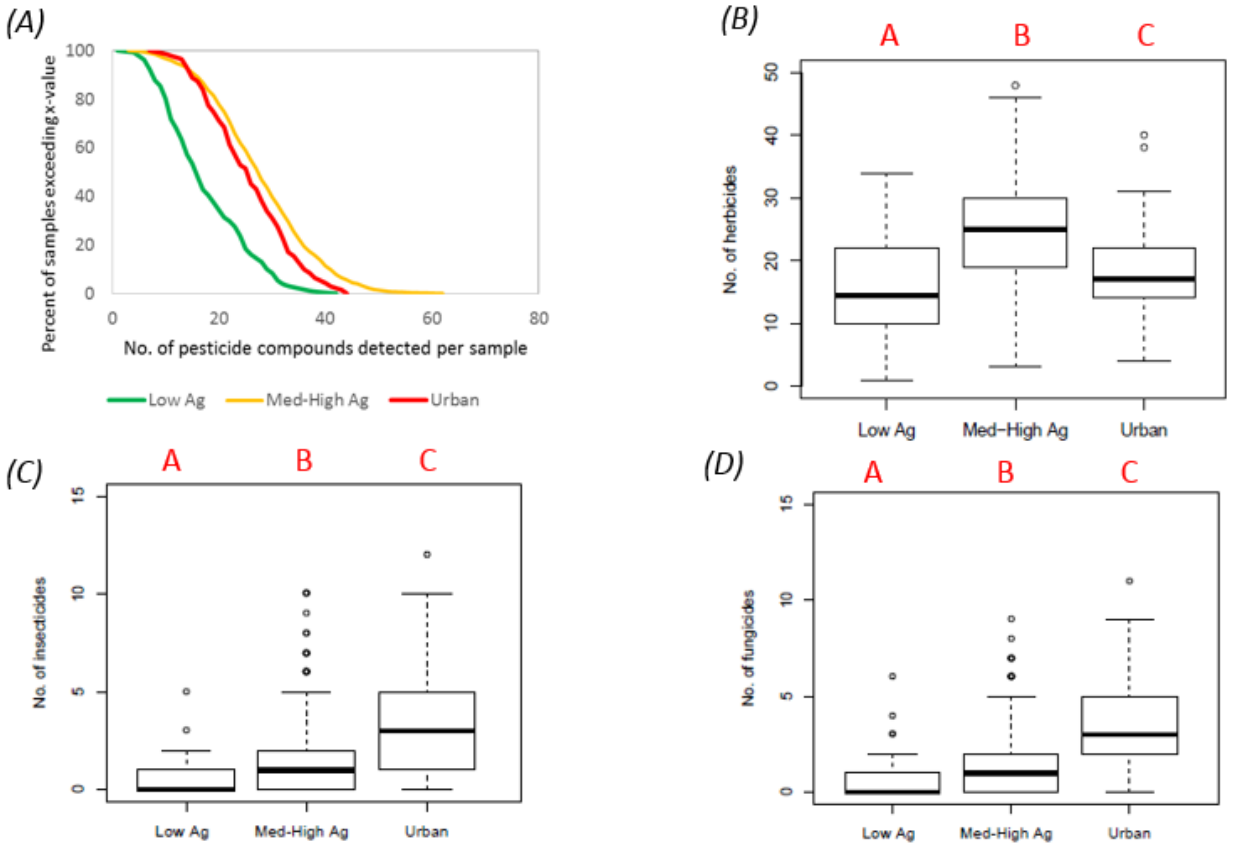


Fig. E.6 shows mixture complexity as a function of land use. Generally, the number of pesticide compounds detected per sample was higher in samples from Med-High Ag and Urban streams than in from Low Ag streams (Fig. E.6A). The number of herbicides detected was significantly higher in Med-Ag streams than other stream types (Fig. E.6B), whereas Urban streams had significantly more insecticides (Figs. E.6C) and fungicides (Figs. E.6D) than Agricultural-gradient streams. Statistical comparisons were made using the nonparametric Kruskal–Wallis test of association and Tukey multiple comparison test ($p<0.05$)

Fig. E.6. Effect of land use on the number of (A) pesticide compounds, (B) herbicides, (C) insecticides, and (D) fungicides detected per sample. Different red letters above the boxplots indicate significant differences ($p<0.05$).



Appendix F. Potential Toxicity: Supplemental Results

F.1. Geographic Distribution of Potential Toxicity

Fig. F.1. Geographic distribution of potential acute toxicity to fish, as determined using the Fish-Pesticide Toxicity Index (PTI). Inset shows proportion of sites with applicable PTI scores in the 0.1 – 1 range and <0.1.

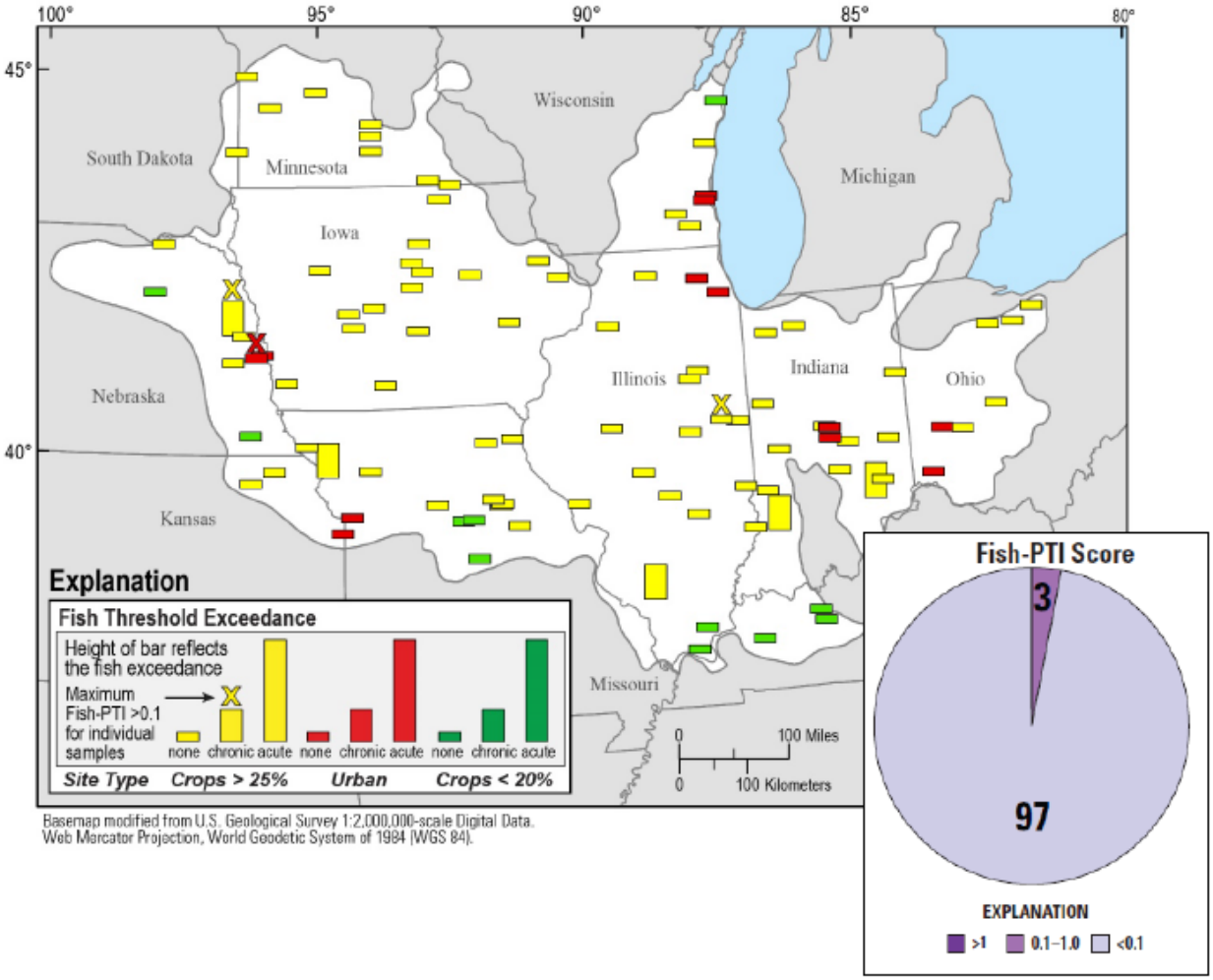


Fig. F.2. Geographic distribution of potential toxicity to aquatic plants at MSQA sites, by land use, as determined using Office of Pesticide Programs Acute Nonvascular Plant (ANVP) benchmarks. Circle size indicates the percentage of samples at each site (of 12 samples per site) that exceeded benchmarks for one or more herbicide classes. X on the circle indicates that the 60-day average atrazine concentration exceeded the USEPA chronic community threshold. Inset shows the proportion of sites in each category of potential toxicity, on the assumption that the likelihood of effects increases with the no. of weeks with exceedance.

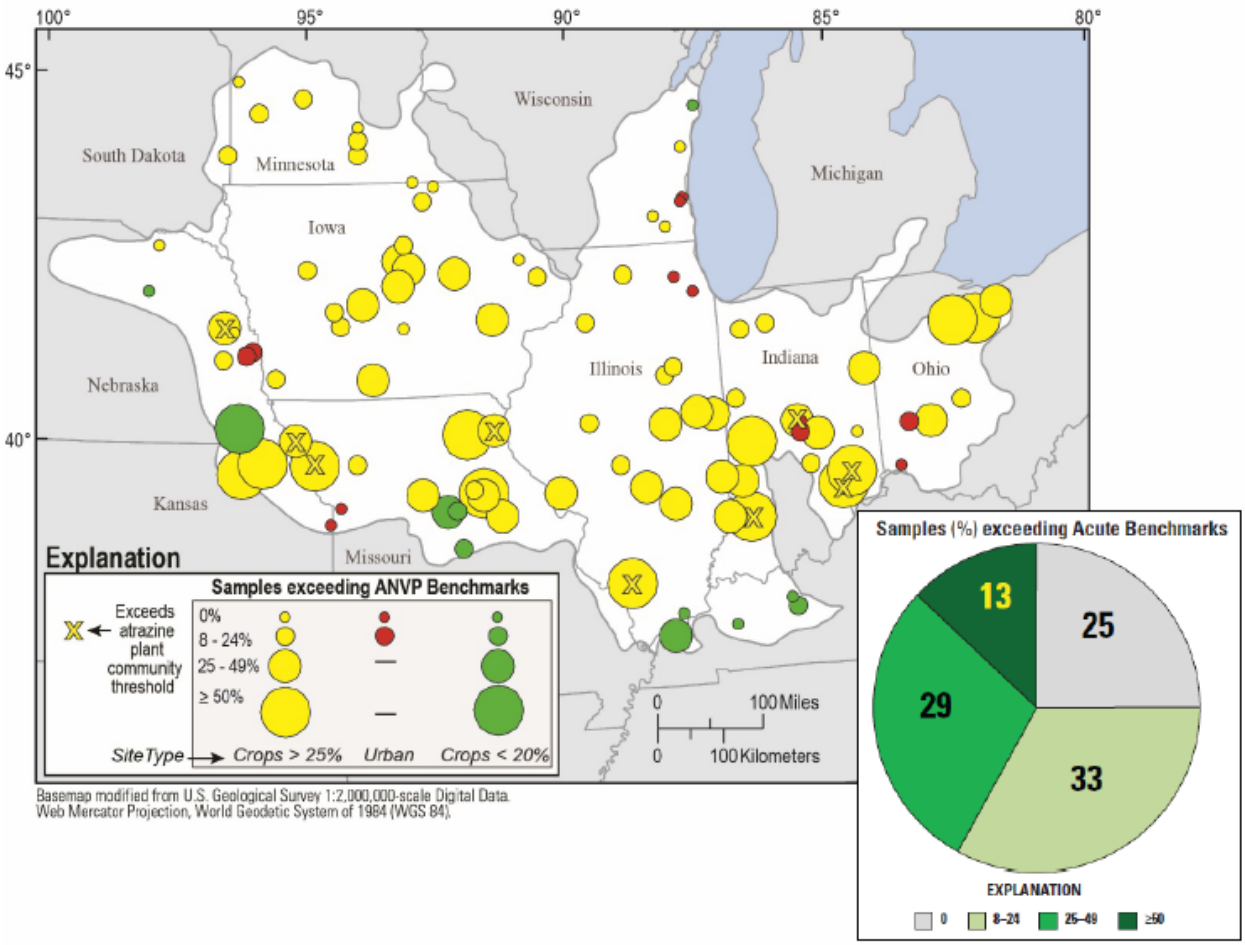
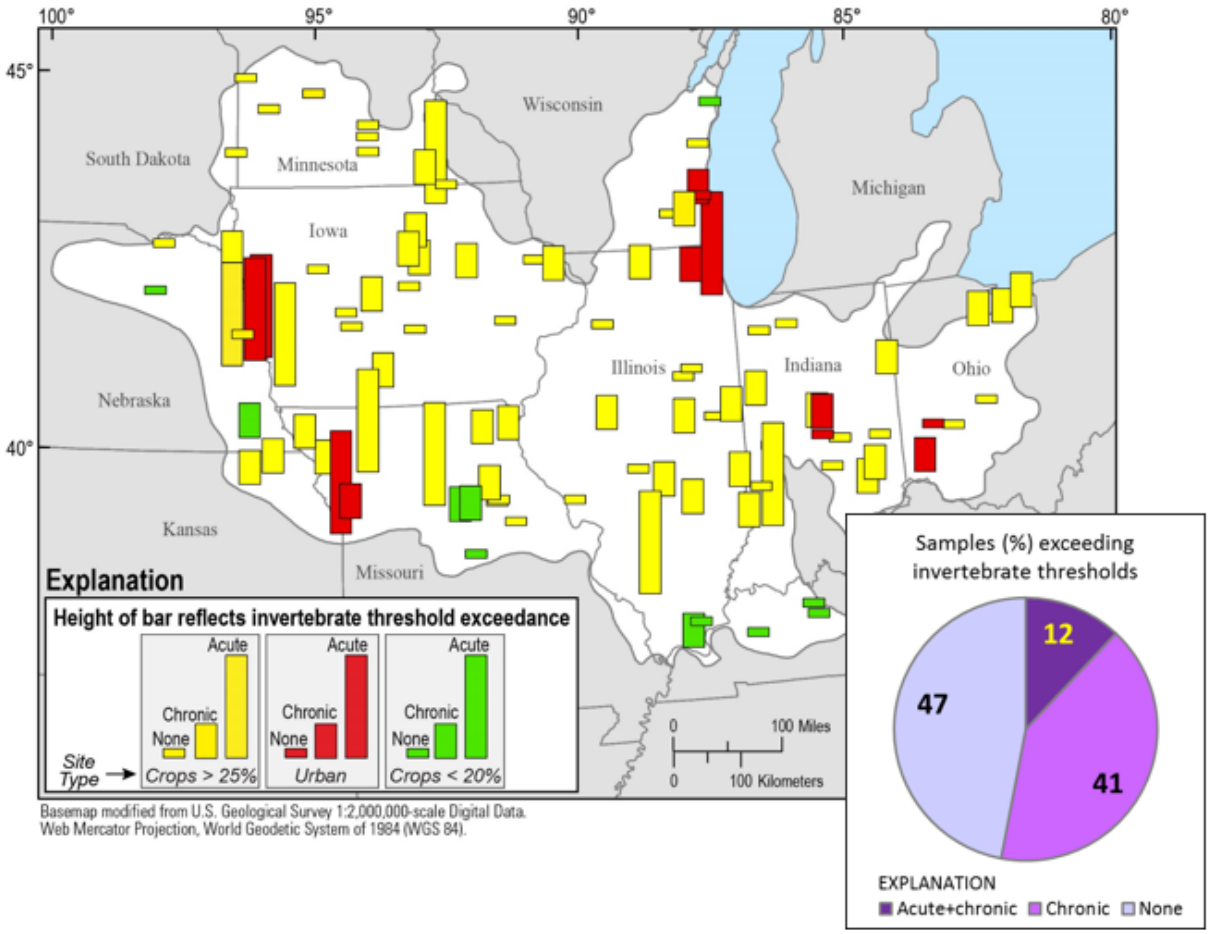


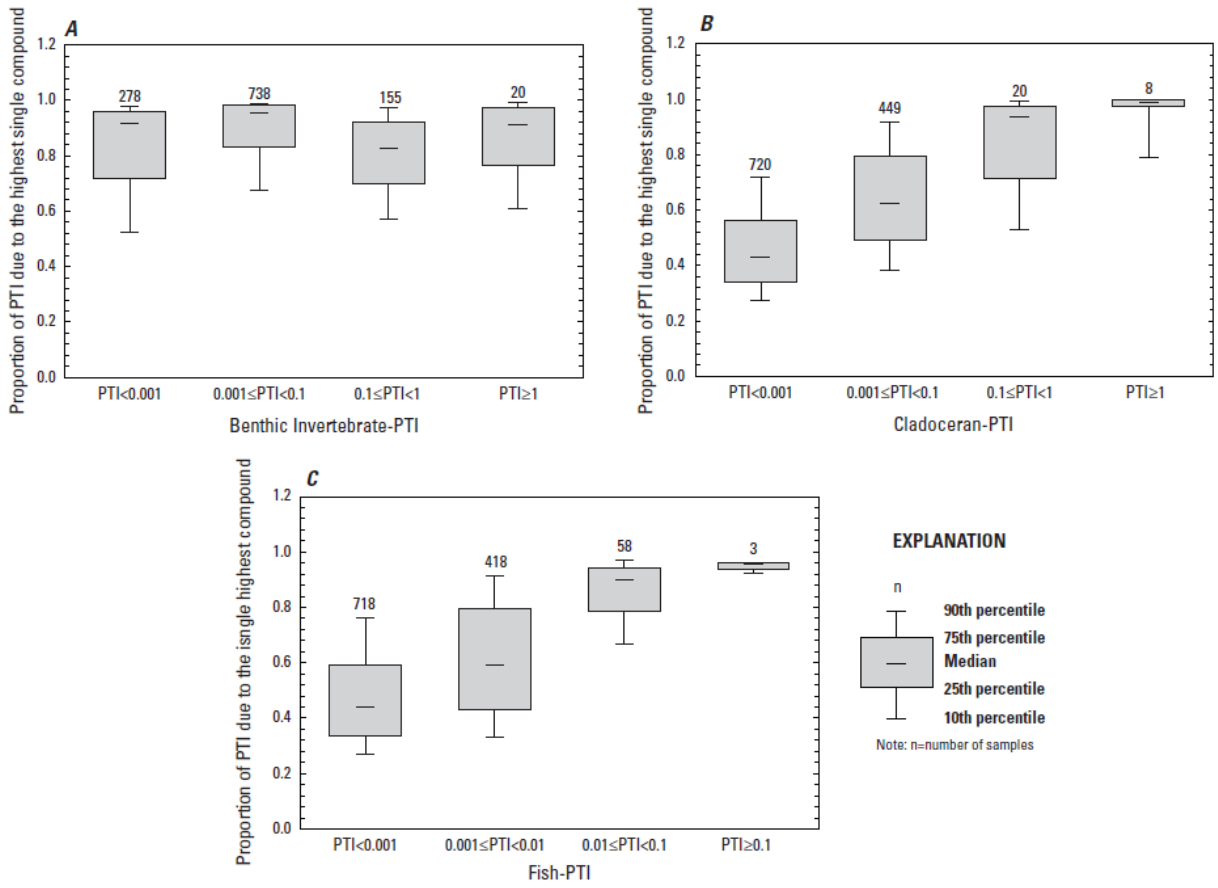
Fig. F.3. Geographic distribution of potential toxicity to invertebrates at MSQA sites, by land use, using a combination of the Cladoceran-Pesticide Toxicity Index (PTI, the Benthic invertebrate-PTI, and Office of Pesticide Program invertebrate benchmarks. Bar height represents the predicted toxicity category for invertebrates at that site: Acute, either a pesticide concentration in one or more samples exceeded the Acute-Invertebrate benchmark or had a PTI score >1 for cladocerans and(or) benthic invertebrates; Chronic, either the 21-day average concentration exceeded the Chronic-Invertebrate benchmark or the 21-day average PTI-Toxic Unit for cladocerans and(or) benthic invertebrates was in the 0.1–1 range for one or more pesticides; None, no acute or chronic thresholds exceeded. Inset indicates the proportion of sites in each category.



F.2. Maximum Toxic Unit in Relation to Pesticide Toxicity Index

For the Benthic Invertebrate-PTI, the TUmax/PTI ratio was >0.7 for 80% of all samples, regardless of the magnitude of the PTI score (Fig. F.4A). For the 175 (15%) samples with Benthic Invertebrate-PTI scores >0.1, the compound most often responsible for the TUmax was imidacloprid (n=152 samples), with the remainder caused by OP insecticides (n=10), atrazine (10), or bifenthrin (3). For cladocerans, unlike for benthic invertebrates, the TUmax/PTI ratio increased as the magnitude of the PTI increased (Fig. F.4B). At very low PTI scores, multiple compounds contributed, but in most (83%) samples that had Cladoceran-PTI scores high enough to have some potential toxicity, the TUmax/PTI was >0.9, indicating that a single compound was responsible. The compound responsible for the cladoceran TUmax was an OP insecticide in 27 of 28 samples (96%); bifenthrin was the sole exception. The TUmax/PTI ratio for fish also increased as the magnitude of the PTI score increased (Fig. 4C), but only 3 samples had PTI scores above 0.1; the compounds responsible for the TUmax were the herbicide acetochlor (n=2) and the fungicide degradate carbendazim (n=1). In summary, in most weekly water samples, especially those with PTI scores > 0.1 (used here as a screening threshold for potential toxicity), a single compound accounted for 80–100% of the PTI.

Fig. F.4. The distribution of the ratio of the maximum Toxic Unit (TUmax) to the Pesticide Toxicity Index (PTI) as a function of PTI score for (A) fish, (B) cladocerans, and (C) benthic invertebrates. A TUmax/PTI ratio >0.9 indicates that a single compound accounts for 90% of the PTI score. [n = no. of samples in each category]


A
Proportion of PTI due to the highest single compound
1.2
1.0
0.8
0.6
0.4
0.2
0.0
278
738
155
20
PTI<0.001
0.001≤PTI<0.1
0.1≤PTI<1
PTI≥1
Benthic Invertebrate-PTI
B
Proportion of PTI due to the highest single compound
720
449
20
8
PTI<0.001
0.001≤PTI<0.1
0.1≤PTI<1
PTI≥1
Cladoceran-PTI
C
Proportion of PTI due to the isngle highest compound
718
418
58
3
PTI<0.001
0.001≤PTI<0.01
0.01≤PTI<0.1
PTI≥0.1
Fish-PTI
EXPLANATION
n
90th percentile
75th percentile
Median
25th percentile
10th percentile
Note: n=number of samples

Appendix G. Quantile Regression: Supplemental Results

Table. G.1. Quantile regression scores for various pesticide stressors in predicting upper quantiles of four types of invertebrate community metrics. (Excel file)

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