Validation of Commercially Available ELISA Assay Kits

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Background

Enzyme-linked immunosorbent assay (ELISA) is a biochemical technique used for detecting specific compounds within environmental samples. This technique can screen and determine pesticides in a large number of samples with minimal sample preparation. ELISA is considered a more cost-effective and rapid technique compared to chemical analysis such as gas chromatography-mass spectrometry (GC/MS) or liquid chromatography-mass spectrometry (LC/MS; Sullivan and Goh, 2000). Although this is a handy method, often ELISA kits are prone to matrix effects – either due to the presence of cross-reactants or nonspecific interferences such as soil moisture and pH (Lesnik, 2000).

Both diuron, a phenylurea herbicide and chlorpyrifos, an organophosphate insecticide are applied for weed control on many crops and are highly used in agriculture in California. This study aims to screen surface water runoff, collected from an alfalfa field, for these two pesticides. In addition, ELISA results will be compared to concentrations determined by either GC/MS or LC/MS analysis.

Previously, the Environmental Monitoring Branch evaluated the sensitivity, repeatability and reproducibility of a commercially available pyrethroid ELISA kit. However, the measurements produced by the kits were highly variable. Thus, this study will continue to validate the potential use of the Abraxis pyrethroid kit for screening environmental samples.

Objectives

The objectives of this study were 1) to validate commercial ELISA pesticide kits for sensitivity, precision, accuracy, matrix effects, and selectivity and 2) to apply these kits to collected irrigation water from an alfalfa field.

Study

- Perform ELISA based on manufactory instructions on spiked and environmental samples.
- Validate ELISA kits for ambient water samples.
- Measure chlorpyrifos and diuron concentrations within irrigation water from an alfalfa field

Procedures

Pyrethroid ELISA Analysis

Pyrethroid test kits were obtained from Abraxis LLC (Warminster, PA); kits were purchased by DPR and transferred to UC Davis. As suggested by the manufacturer's instructions, kits were stored at 4°C until use. Sample analysis was conducted as instructed by the supplied test kit insert (described below).

- <u>Reagents and Equipment</u>

-Pyrethroid Antibody Coupled Paramagnetic Particles
-Pyrethroid Enzyme Conjugate (horseradish peroxidase (HRP) labeled Permethrin analog)
-Permethrin Standards (0.75, 2.5, 5.0, 15.0 ppb)
-Control (approx. 3.0 ppb of Permethrin)
-Diluent/Zero Standard (without any detectable Permethrin)
-Color Solution (hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine)
-Stopping Solution (diluted sulfuric acid (0.5%))
-Washing Solution (deionized water)
-Test Tubes (100 tubes)
-Vortex mixer
-Multi-volume precision and repeating pipettes (deliver 250-500 uL and 1000 uL)
-Magnetic separation rack
-Photometric analyzer (reads at 450nm)

- <u>Procedure</u>

1. Label glass test tubes for each standard, control, and samples as follows:
   1, 2-- Diluent/Zero Standard, 0 ppb
   3, 4-- Standard 1, 0.75 ppb
   5, 6-- Standard 2, 2.5 ppb
   7, 8-- Standard 3, 5.0 ppb
   9, 10-- Standard 4, 15.0 ppb
   11-- Control
   12-- Sample 1
   13-- Sample 2
   14-- Sample 3
   ***continue labeling until all samples have been included*

2. Add 250 uL of the appropriate standard, control, or sample to the test tube.
3. Mix the Pyrethroid Antibody Coupled Paramagnetic Particles thoroughly and add 500 uL to each tube.
4. Vortex for 1 to 2 seconds minimizing foaming.
5. Incubate for 20 minutes at room temperature.
6. Add 250 uL of Pyrethroid Enzyme Conjugate to each tube.
7. Vortex for 1 to 2 seconds minimizing foaming.
8. Incubate for 30 minutes at room temperature.
9. Separate in the Magnetic Separation Rack for 2 minutes.
10. Decant and gently blot all tubes briefly in a consistent manner.
11. Add 1 mL of Washing Solution to each tube and vortextubes for 1-2 seconds. Return tubes and allow to remain in the magnetic separation unit for two (2) minutes.
12. Decant and gently blot all tubes briefly in a consistent manner.
13. Repeat Steps 12 and 13 an additional time.
14. Remove the rack from the separator and add 500 uL of Color Solution to each tube.
15. Vortex for 1 to 2 seconds minimizing foaming.

16. Incubate for 30 minutes at room temperature.
17. Add 500 uL of Stopping Solution to each tube.
18. Add 1 mL Washing Solution to a clean test tube. Use as the blank.
19. Read results at 450 nm within 15 minutes after adding the Stopping Solution.

Chlorpyrifos ELISA Analysis

Chlorpyrifos test kits were obtained from Modern Water Inc. (Newark, DE); kits were purchased by DPR and transferred to UC Davis. As suggested by the manufacturer's instructions, kits were stored at 4°C until use. Sample analysis was conducted as instructed by the supplied test kit insert (described below).

- Reagents and Equipment

- 8 Antibody-coated strips (12 wells each) per strip holder
- 1.0 ppm Chlorpyrifos-ethyl stock solution
- Chlorpyrifos enzyme conjugate
- Substrate
- Stopping Solution (1N HCl)
- Deionized water
- 100 mL volumetric flasks
- Multi-volume precision and repeating pipettes (deliver 10, 100, and 1000 uL)
- glass tubes/ vials
- timer
- microtiter plate or strip reader

- Procedure

1. Prepare the chlorpyrifos-ethyl calibration standards (0.05, 0.3 and 1 ppb) from the 1.0ppm standard.
2. Add 100 uL of the negative control, each calibrator (0.5-1 ppb) and each sample to the individual wells.
3. Add 100 uL of the chlorpyrifos enzyme conjugate to each well (use of a multi-volume pipette is suggested).
4. Thoroughly mix by moving the plate in a circular motion.
5. Cover the wells with parafilm or a plate cover and incubate for 1 hour at room temperature. Mixing at approx 200 rpm is desired during incubation, but not required.
6. Following incubation, shake the contents out of the plate into a sink.
7. Fill the plates with cool tap water, shake, and empty. Repeat washing the wells 5 times. Tap the remaining water out.
8. Add 100 uL of Substrate to each well.
9. Cover the wells and incubate for 30 minutes at room temperature. Mixing at approx 200 rpm is desired during incubation, but not required.
10. Add 100 uL of Stop solution to each well and mix (solutions will turn yellow).
11. Read the plate at 450nm within 30 minutes of adding the stop solution.

Diuron ELISA Analysis

Diuron test kits were obtained from Abraxis LLC. (Warminster, PA); kits were purchased by DPR and transferred to UC Davis. As suggested by the manufacturer's instructions, kits were stored at 4°C until use. Sample analysis was conducted as instructed by the supplied test kit insert (described below).

- Reagents and Equipment

- 8 Antibody-coated strips (12 wells each) per strip holder
- 5 diuron standards, positive and negative controls
- 5X washing buffer
- Enzyme conjugate
- Substrate
- Stopping Solution (diluted sulfuric acid)
- 500 mL volumetric flask
- Multi-volume precision and repeating pipettes (deliver 25, 50, and 100 uL)
- timer
- orbital shaker
- microtiter plate or strip reader

- Procedure

1. Dilute the washing buffer 5-fold (100mL of 5X buffer and 400mL DI water).
2. Add 25 uL of the assay buffer into each well (use of a multi-volume pipette is suggested).
3. Add 50 uL of the standards, controls and samples to individual wells (duplicates or triplicates is desired).
4. Add 50uL of the enzyme conjugate to each well (use of a multi-volume pipette is suggested).
5. Thoroughly mix by moving the plate in a circular motion.
6. Cover the wells with parafilm or a plate cover and incubate for 30 minutes at room temperature. Mixing on an orbital shaker is desired, but not required.
7. Following incubation, shake the contents out of the plate into a sink.
8. Wash the strips 3 times with the 1X wash buffer (300uL each well), shake, and empty. Repeat washing the wells 3 times. Tap the remaining water out.
9. Add 100 uL of Substrate/ color solution to each well.
10. Cover the wells and incubate for 25-30 minutes at room temperature in the dark. Mixing is desired, but not required.
11. Add 50 uL of Stop solution to each well and mix (solutions will turn yellow).
12. Read the plate at 450nm.

Data Analaysis

- Calculations

1. Calculate the mean absorbance for the standards and samples.

2. The %Bo is used to equalize different runs of an assay, thus the variations of controls and samples between runs are normalized (more constant) by the negative control.

$$B/Bo = \%B = \frac{\text{avg. absorbance of calibrator or sample}}{\text{avg. absorbance of standard zero}} * 100\%$$

2. Graph the %Bo for each calibrator against each concentration (log scale).

3. Determine the diuron concentration for each sample by interpolation using the graph.

4. Sample concentration can only be determined in the %Bo falls within the range of the graph. If determined to be higher, then the sample must be diluted and run the assay again.

Results and Discussion

After samples were analyzed, their concentrations were determined based on calculations provided in each test kit. Using a calibration curve, within the range of each individual kit, measured concentrations were obtained. If samples were diluted to be measured within the test kit range, the dilution factor was applied to the calculated concentration.

Pyrethroid ELISA

For the pyrethroid ELISA kit, water samples spiked at different concentrations of permethrin cis or trans isomers were analyzed and the percent recovery was calculated. As shown in Table 1, the recovery at each concentration was higher than expected and each %CV was greater than 10. These results indicate that the kit could not accurately determine a known spiked concentration. In addition, the error between measured absorbencies (%CV) was large, thus reproducibility between replicates was highly variable. Furthermore, day to day variation also resulted (Table 2); repeatability was minimal.

According to the manufacturer's insert, this kit has been found to cross react with pyrethroid analogues at an identified least detectable dose (LDD; Table 3). To confirm this, cypermethrin, lambda-cyhalothrin and permethrin were analyzed at a spiked concentration of 100 ppb (Table 4). The kit proved to measure other pyrethroids although measured concentrations varied from day-to-day and percent recoveries were low. At lower concentrations (5 and 10 ppb) variable measurements also resulted.

Although this kit can accommodate many samples, the variability between runs and lack of reproducibility made it an unreliable source of analysis. There are many steps that could have contributed to variable results, including pipette errors, the magnetic particles not being completely mixed, insufficient vortex mixing, and others; however it is unclear which may have contributed. Due to the inconsistency of the kits, it was decided that they no longer be validated and thus determined they are not a reliable source of pyrethroid analysis in environmental samples.

Chlorpyrifos ELISA

For the chlorpyrifos ELISA kit, water samples collected from two irrigated alfalfa fields, was used to validate and measure concentrations in runoff. Water samples were collected and analyzed up to a few months after the initial chlorpyrifos application. As shown in Table 5, chlorpyrifos concentrations decreased in the runoff to levels below the test kit's measurement concentration of 0.05 ppb; decreases were observed for both fields. The introduced error between absorbency readings (%CV) were relatively low (<10) compared to the pyrethroid kit. For each analysis, concentrations were relatively similar although samples were collected during a timed event, up to a few hours following initiation of runoff.

To compare concentrations obtained via ELISA analysis, five samples from each field, collected in May, were sent to the Water Pollution Control Laboratory (WPCL; CA Dept. of Fish and Game). There, samples were subjected to extraction and gas chromatography analysis. Results (Table 6) show the ELISA data to report higher concentrations than reported by WPCL; concentrations differed by 73-227%. Although, this is a large difference in concentrations, it could be attributed to lack of sample preparation prior to ELISA analysis; thus whole water without solvent extraction is used. The impact between filtered and un-filtered field samples was investigated (Table 7). By filtering samples, to remove suspended solids and other debris, the concentration of chlorpyrifos decreased. Sample concentrations between the two had a difference of 15-156%. However, since it was desired to analyze the water as a whole, filtration was no longer used.

According to the manufacturer's insert, this kit has been found to cross react with other pesticides with an identified lower limit of detection (LLD; Table 8); such pesticides were not investigated here. Overall, the tests conducted using the chlorpyrifos ELISA kit proved to be a rapid and reproducible technique. Although concentrations varied between ELISA and GC, these kits would be a good way to screen samples for the presence of chlorpyrifos and estimate concentrations.

Diuron ELISA

For the diuron ELISA kit, water samples collected from two irrigated alfalfa fields, was used to validate and measure concentrations in runoff. Data from three irrigation events is presented in Table 9. Samples collected during the first event had high concentrations of diuron as measured by ELISA and LC/MS, however, the test kit did not measure concentrations at the high level LC/MS did. Furthermore, concentrations varied between fields with Field B having lower concentrations; this was consistent for both analyses. It is unclear why the two fields varied so dramatically and why the LC/MS analysis of Field B was significantly lower than Field A. For the second irrigation event, both analyses resulted in similar concentrations. However, concentrations of diuron measured by LC/MS, within Field B waters, was very similar to the concentrations from the first irrigation. Concentrations drastically decreased by the third irrigation event for both fields; Field B concentrations still remained low. For each irrigation event, the % difference between the ELISA and LC/MS measurements were highly variable

ranging anywhere from -49 to 170%. The decrease in concentrations shows that diuron was being washed off of the field during irrigation and was degrading over time.

To determine interference between other applied pesticides potentially present in the field runoff, a spike recovery test was conducted. Triplicate water samples were collected from an alfalfa field previously sprayed with diuron and one without diuron. Each sample was spiked with diuron for a final concentration of 0.3ppb. The experimental field water spike had variable recoveries, ranging from 76-122% whereas, the non-experimental water spike had a recovery near 100 % for all three samples (Table 10). The experimental field had a measurable amount of diuron prior to spiking, thus the calculated spike concentration was higher than 0.3ppb. A difference in the two field spike recovery tests showed that there was no background interference from other fields when analyzing experimental field water. In addition, this test shows good repeatability for the test kit when using uncontaminated water. ELISA and LC/MS analysis was conducted on the experimental field water only and both detected diuron within the water at similar concentrations (Table 11). According to the manufacturer's insert, this kit has been found to cross react with other pesticides with an identified percent of cross-reactivity (CR; Table 12); such pesticides were not investigated with this kit. Although it is unknown if any of the compounds listed in Table 12 have previously been applied to the surrounding alfalfa fields, it is possible that background interference would have been determined for the above mentioned samples.

Overall, the tests conducted using the diuron ELISA kit proved to be a rapid and reproducible technique. Although concentrations slightly varied, for the first irrigation event, between ELISA and LC/MS, these kits are a good way to screen samples for the presence of diuron and estimate concentrations.

Conclusion

The Abraxis pyrethroid test kit, when performed by the manufacturer's instructions was not reproducible or repeatable. Calibration curves varied for each analysis and high %CVs resulted for each sample and calibrator. In addition, the test kit results were skewed by interferences from other pesticides and matrix effects. This test kit was not a reliable tool. However, both the Strategic Diagnostics chlorpyrifos and Abraxis diuron test kits performed well. Both kits were reproducible and results were more accurate. Both kits measured the pesticides at concentrations similar to analytical methods (GC/MS and LC/MS). It was found that test kits containing 96-well plates were more accurate than the kits with test tubes. Overall, these kits proved ELISA to be a rapid screening technique for many samples.

References

Lesnik, B. 2000. Immunoassay techniques in environmental analyses. Encyclopedia of Analytical Chemistry. R.A. Meyers Ed. Pp. 2653-2672.

Sullivan, J.J and K.S. Goh. 2000. Evaluation and validation of a commercial ELISA for diazinon in surface waters. J Agric Food Chem 48: 4071-4078.

Table 1. Pyrethroid ELISA kit spike recovery for one trial.

Compound	Spiked concentration (ppb)	Measured Concentration (ppb)	Percent Recovery (%)	%CV
cis-Permethrin	0.5	1.78	356.07	10.08
	1	1.72	171.68	10.68
	2.5	3.95	157.99	4.84
	5	7.20	144.02	18.57
	10	18.27	182.72	21.11
	15	23.35	155.64	26.68
trans- Permethrin	0.5	2.26	452.72	6.95
	1	3.17	316.59	12.83
	2.5	6.23	249.37	7.32
	5	16.94	338.76	10.85
	10	22.29	222.87	25.30
	15	41.18	274.57	12.13

Table 2. Pyrethroid ELISA kit day-to-day variability.

Compound	Spiked concentration (ppb)	Measured Concentration (ppb)	Percent Recovery (%)	%CV
Oct. 21, 2011				
cis-Permethrin	10	17.88	178.79	6.68
trans- Permethrin	10	33.48	334.81	14.60
Oct. 24, 2011				
cis-Permethrin	10	7.37	73.66	8.68
trans- Permethrin	10	12.08	120.79	27.61
Oct. 26, 2011				
cis-Permethrin	10	15.36	153.56	9.96
trans- Permethrin	10	19.35	193.48	27.26

Table 3. Cross reactivity of pyrethroid analogues as determined by Abraxis.

Compound	LDD (ppb)
permethrin	0.750
cypermethrin	4.75
lambda-cyhalothrin	9.2
bifenthrin	13.5
resmethrin	200
cyfluthrin	220
tetramethrin	>1000
3, PBA	170

Table 4. Cross reactivity of two pyrethroid analogues and their day-to-day variability.

Compound	Spiked concentration (ppb)	Measured Concentration (ppb)	Percent Recovery (%)	%CV
Oct. 21, 2011				
Cypermethrin	100	62.89	62.89	27.79
lambda-cyhalothrin	100	43.57	43.57	31.96
Oct. 24, 2011				
Cypermethrin	100	11.70	11.70	10.62
lambda-cyhalothrin	100	7.71	7.71	96.78
Oct. 26, 2011				
Cypermethrin	100	20.07	20.07	33.45
lambda-cyhalothrin	100	20.73	20.73	10.04

Table 5. Change in chlorpyrifos concentrations during three irrigation events after initial application.

Sample	Measured Concentration (ppb)	%CV
May 21, 2012		
Field Blank	0.036	2.77
A S1	0.433	0.70
A S2	0.612	4.91
A S3	0.560	5.36
A S4	0.563	4.45
A S5	0.543	5.48
A S6	0.538	2.07
A S7	0.564	6.72
A S8	0.570	5.04
A S9	0.631	12.63
A S10	0.575	1.20
B S1	0.590	7.08
B S2	0.515	7.21
B S3	NA	NA
B S4	0.562	6.33
B S5	0.523	11.36
B S6	0.508	6.45
B S7	0.618	2.41
B S8	0.565	3.98
B S9	0.620	2.29
B S10	0.587	2.21

Sample	Measured Concentration (ppb)	%CV
June 15/16, 2012		
Field Blank	ND	3.87
A S1	0.447	6.96
A S2	0.376	4.12
A S3	0.461	8.95
A S4	0.373	3.03
A S5	0.350	5.41
A S6	0.333	2.01
A S7	0.362	12.74
A S8	0.352	2.66
A S9	0.308	6.01
A S10	0.206	3.73
B S1	0.306	7.78
B S2	0.342	9.98
B S3	0.373	2.37
B S4	0.318	5.63
B S5	0.313	3.61
B S6	0.363	3.32
B S7	0.356	3.44
B S8	0.303	5.91
B S9	0.256	4.47
B S10	0.362	8.54

Sample	Measured Concentration (ppb)	%CV
Aug. 30, 2012		
Field Blank	ND	1.00
A S1	ND	0.83
A S2	ND	6.95
A S3	ND	0.70
A S4	ND	1.61
A S5	ND	0.86
A S6	ND	6.55
A S7	ND	1.33
A S8	ND	0.88
A S9	ND	2.34
A S10	ND	1.00
B S1	ND	0.64
B S2	ND	9.09
B S3	ND	2.14
B S4	ND	1.84
B S5	ND	1.91
B S6	ND	1.95
B S7	ND	0.37
B S8	ND	2.86
B S9	ND	6.62
B S10	ND	1.58

Table 6. Chlorpyrifos concentrations, within runoff, measured via gas chromatography and ELISA.

Sample (Field A or B)	Concentration by GC analysis	Concentration by ELISA analysis	Difference (%)
A S1	0.11	0.43	209.9
A S2	0.25	0.61	144.0
A S5	0.26	0.54	107.7
A S7	0.26	0.56	115.4
A S10	0.29	0.57	96.6
B S1	0.18	0.59	227.8
B S2	0.20	0.51	155.0
B S5	0.25	0.52	108.0
B S7	0.26	0.62	138.5
B S10	0.34	0.59	73.5

Table 7. Chlorpyrifos concentrations in filtered and un-filtered field water.

Un-filtered Sample (Field A)	Measured Concentration (ppb)	Filtered Sample (Field A)	Measured Concentration (ppb)	Difference (%)
A S1	0.433	A S1	0.377	14.9
A S2	0.612	A S2	0.346	76.9
A S3	0.560	A S3	0.218	156.9
A S4	0.563	A S4	0.288	95.5
A S5	0.543	A S5	0.232	134.1
A S6	0.538	A S6	0.303	77.6
A S7	0.564	A S7	0.242	133.1
A S8	0.570	A S8	0.445	28.1
A S9	0.631	A S9	0.284	122.2
A S10	0.575	A S10	0.273	110.6

Table 8. Cross reactivity (CR) to chlorpyrifos analogues as the lower limit of detection (LLD).

Compound	LLD (ppb)
chlorpyrifos-ethyl	0.05
chlorpyrifos-methyl	0.02
3,5,6-trichloro-2-pyridinol	100
azinophos	10
azinophos-methyl	82
bromophos	0.5
bromophos-methyl	0.07
fenchlorphos	0.01
fenitrothion	40
parathion-methyl	50
picloram	1000
quinalfos	20
triazophos	2
triclopyr	3

Table 9. Diuron concentrations measured in runoff from three irrigation events by ELISA and LC/MS.

Sample (Field A or B)	Measured Conc. (ppb) ELISA	Measured Conc. (ppb) LC/MS	Difference (%)
Feb.26/ 27, 2013			
A S1	161.3	203.1	-20.6
A S3	136.2	168.5	-19.2
A S5	100.1	153.2	-34.7
A S7	111.0	171.2	-35.1
A S9	106.1	208.8	-49.2
B S1	106.2	46.0	130.9
B S3	83.6	42.5	96.6
B S5	80.3	36.4	120.6
B S7	76.8	37.8	103.4
B S9	81.9	30.3	170.3

Sample (Field A or B)	Measured Conc. (ppb) ELISA	Measured Conc. (ppb) LC/MS	Difference (%)
Mar.21/ 22, 2013			
A S1	66.5	60.2	10.5
A S3	59.7	43.0	38.8
A S5	36.4	36.5	-0.3
A S6	35.5	28.9	22.8
A S7	43.9	31.7	38.5
A S9	43.9	43.8	0.2
B S1	41.6	38.6	7.7
B S3	29.9	33.0	-9.3
B S5	22.3	23.4	-4.8
B S7	34.7	21.2	63.5
B S9	36.3	30.7	18.4

Sample (Field A or B)	Measured Conc. (ppb) ELISA	Measured Conc. (ppb) LC/MS	Difference (%)
Apr. 26/ 29, 2013			
A S1	13.6	14.5	-6.3
A S3	8.8	10.8	-18.5
A S5	9.3	8.4	10.3
A S6	9.3	10.0	-6.7
A S7	12.1	10.2	18.1
A S10	14.9	14.9	-0.3
B S1	9.5	8.2	15.5
B S3	9.0	7.4	22.0
B S4	8.8	6.9	26.7
B S5	9.0	7.3	23.9
B S7	10.3	8.4	22.0
B S9	8.5	9.6	-11.9

Table 10. Determination of background interference as measured in spiked (0.3ppb) experimental and non-experimental field runoff.

Sample (Experimental Field 10x dilution)	Measured Concentration (ppb) ELISA	Measured Spiked Concentration (ppb)	Percent Recovery (%)
May 23/27, 2013			
Expt Field Rep1	12.75	11.97	93.9
Expt Field Rep2	7.09	8.65	122.1
Expt Field Rep3	11.9	9.13	76.7

Sample (Non-Experimental Field)	Measured Concentration (ppb) ELISA	Percent Recovery (%)
Non-expt Field Rep1	0.31	103.3
Non-expt Field Rep2	0.33	108.6
Non-expt Field Rep3	0.34	112.5

Table 11. Determination of background interference in experimental field runoff determined by ELISA and LC/MS.

Sample (Experimental Field 10x dilution)	Measured Concentration (ppb) ELISA	Measured Concentration (ppb) LC/MS	% difference
May 23/27, 2013			
Expt Field Rep1	10.19	9.36	8.87
Expt Field Rep2	6.29	5.83	7.89
Expt Field Rep3	5.8	6.52	-11.04

Table 12. Cross reactivity (CR) to diuron analogues:

Compound	CR (%)
diuron	100
neburon	1250
chlorbromuron	62.5
linuron	25
chlortoluron	7.8
propanil	4.8
monuron	<1
monolinuron	<1
fenuron	<1
bromuron	<1
iIsoproturon	<1
propham	<1