Pesticides in Drinking Water: Project-Based Learning within the Introductory Chemistry Curriculum

Patricia B. O'Hara* and Jon A. Sanborn
Department of Chemistry, Amherst College, Amherst, MA 01002-5000; **pbohara@amherst.edu*

Meredith Howard
Department of Chemistry, California Institute of Technology, Pasadena, CA 91125

Several recent studies of the high attrition rates in introductory science courses suggest that part of our students' lack of excitement comes from our failure to point out the importance of the science they are learning to real-world problems (*1*). Unfortunately, a promise that relevance will be made clear at some point in the future is not sufficient to prevent discouraging some very able students from pursuing studies in science. Studies have shown that some groups, women and minority students in particular, can be motivated to learn material when it is put in a context in which they see its relevance (*2*). It is also our observation that students are particularly motivated when they have access to state of the art instruments and techniques in the laboratory.

Background

We have developed a four-week laboratory module that focuses on the analysis of trace levels of xenoestrogenic pesticides in local drinking water and a comparison of the molecular shapes of these pesticides to the hormone estrogen. The importance of understanding atomic and molecular structure is motivated by the goal of understanding the interactions of molecules such as natural hormones and their receptors, as well as potentially toxic synthetic estrogen competitors, the xenoestrogens, which have been implicated in causing cancer and infertility.

Concern for the existence of low levels of xenoestrogens in ground water is related to their potentially detrimental health effects, such as a possible causative role in breast cancer (*3, 4*). Congress has responded to public concern by proposed amendments to the Clean Water Act, first by the 101st Congress (H.R. 599 & 3574) and then by the 103rd Congress (H.R. 2898, 1114 & 2093) which require all communities to test and report levels of xenoestrogens in public drinking water by the year 2000.

Since Amherst College is located in an agricultural belt of the Connecticut River Valley, xenoestrogenic ground-water contaminants, if present, may be linked to past and present use of pesticides in farming. We decided to focus on pesticides used in tobacco farming: namely, endosulfan, methoxychlor, and, because of its persistence, the DDT family. Our introductory chemistry course in atomic and molecular structure uses this local environmental concern as a backdrop for presentation of some basic introductory concepts: conservation of mass, techniques of mass determination, solution properties, quantitative determination of concentration using absorption spectroscopy, determination of molecular shape using VSEPR, resonance, and models of conformational flexibility. Additionally, students are introduced to computer molecular modeling. This paper details how we adapted the laboratory component of the class to be in line with our pedagogical motivator, pesticide levels in drinking water, so that students can actually contribute real answers to this real-world problem. We have used this module for two semesters.

Students collected water samples from spring-fed reservoirs, natural artesian wells, and private wells as part of the first laboratory session. The subsequent three laboratory weeks involved the extraction of pesticides from a portion of each sample using solid-phase extraction (SPE) and GC–MS analysis. The remainder of the samples were then tested using two ELISAs (enzyme linked immunosorbent assay): one designed for water/soil analysis of DDT and one that tests for endosulfan and several pesticides of the cyclodiene family. The final week of the module was devoted to molecular modeling of the pesticides and their comparison with the structures of the biologically relevant estrogen hormones whose action they mimic and also with structures derived from VSEPR, hybridization, and resonance. A component of the laboratory module that was not implemented the first year is the development of a database to determine the statistical validity of the sampling. The database will provide a checkpoint for determining if errors in sampling and testing occurred and if additional sampling is needed. Once established, a group report will be shared with the local water commissioner and ultimately the community to emphasize the collaborative aspect of science as well as the significance of the students' findings to a real-world issue.

Water Sampling and Initial Sample Characterization

A survey was taken of community interest in the results of the project. We obtained maps of the wells drilled in the Lawrence Swamp, which supplies the drinking water to the Amherst College campus. Several artesian sites with easily accessible sampling wells were located and the relatively simple equipment needed to follow EPA guidelines for sample collection was either purchased or borrowed. We also sampled water from an above-ground reservoir, Atkins reservoir, and one private well in North Amherst.

On the first day of lab for each lab section, students were divided into two groups of 12. Each group was transported to the several water source locations for the Town of Amherst. At each water source site, appropriate sampling instructions were given and the students then collected approximately 500 mL of water. They also measured and recorded temperature, pH, and conductivity. Laptop computers would be particularly useful for both laboratory and field work for this experiment.

Experimental Procedures

Materials

The approximate cost per student pair is in parentheses.

100 mg Accubond OD, J&W Scientific, octydecyl-C18; 3 columns per student pair ($2.80)

Ethyl acetate, 99.5+% PRA grade, CAS 141-78-6; 12 mL per student pair ($0.17)

Methyl alcohol, 99.9+% PRA grade, CAS 67-56-1; 3 mL per student pair ($0.03)

Envirogard DDT soil/water kit #73100, Strategic Diagnostics, Inc. ($10/test) *or*

Envirogard Cyclodiene test kit #73300, Strategic Diagnostics, Inc. ($10/test)

Preparing the GC–MS Sample

Solid-phase extraction (SPE) of pesticides and other organic compounds was accomplished using a 100-mg Precision Extraction Cartridge equipped with a syringe adaptor. Ethyl acetate was used to extract the bound organics from the C18 column (*5*). The column (60 mm × 10 mm) was pretreated by passing first 3 mL of ethyl acetate and then 1 mL of dry methanol through it, using a syringe, until the liquid level was just above the white adsorbent. Trapped air was removed by inverting the column while forcing liquid through with the syringe. One hundred milliliters of sample water was passed through the column at a rate of ~25 mL/min, ensuring that the adsorbent always remained covered with water. After the 100-mL sample had been passed through the column, the column was dried thoroughly using a vacuum or aspirator to draw air through it for at least five minutes.[1] The organic residue bound to the column was then extracted with 1 mL of ethyl acetate. The first 100 μL of ethyl acetate eluted, which contains the bulk of the extracted pesticides, was collected into a microfuge tube and the exact volume collected was recorded.[2]

The instrument specifications are given in Table 1. A gas chromatograph–mass spectrometer was used for separation of chlorinated pesticides (*6*). A standard mixture was prepared, which contained 1 ppm (mg/L) each of DDT, DDD, DDE, methoxychlor (Aldrich), and endosulfan (VWR).[3] Each pair of students injected 3 μL of their 100 μL ethyl acetate sample. Figures 1 through 4 show the results for a known standard mix and the three tested drinking water sources. The mass spectrum of a control sample of DDT was given to each student in prelab lecture and the students were asked to explain the observation of weak peaks at *m/z* 354.49 (parent compound)

Table 1. Instrumental Conditions for Determination of Pesticides in Drinking Water by GC–MS

Instrument/Parameter	Specification
Gas chromatograph–Mass spectrometer	Hewlett Packard 5890/5972 with EI detector
Column	DB-1701, 15 m × 0.25 mm
Oven program	2 min at 190 °C 0 °C/min ramp to 250 °C hold for 3 min 40 °C/min ramp to 280 °C hold for 3 min
Injector temperature	250 °C
Detector temperature	280 °C
Flow rate	0.89 mL/min

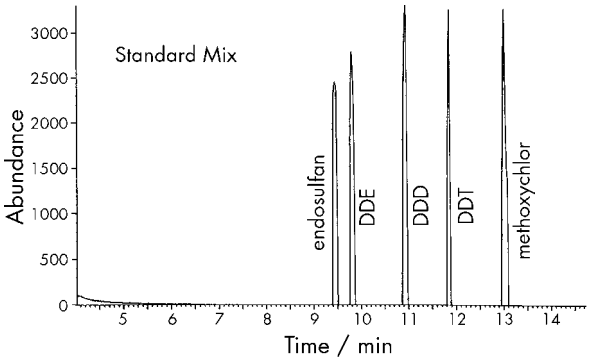


Figure 1. GC of 5-pesticide mix.

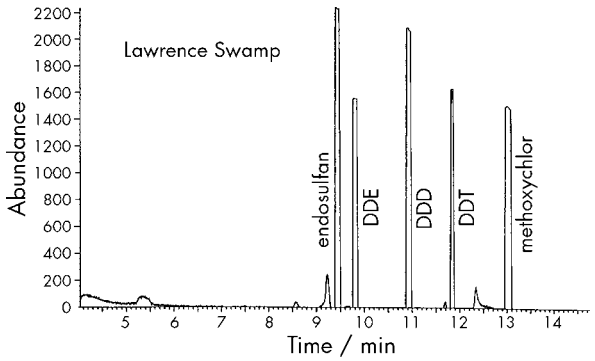


Figure 2. Representative GC of the Lawrence Swamp water source.

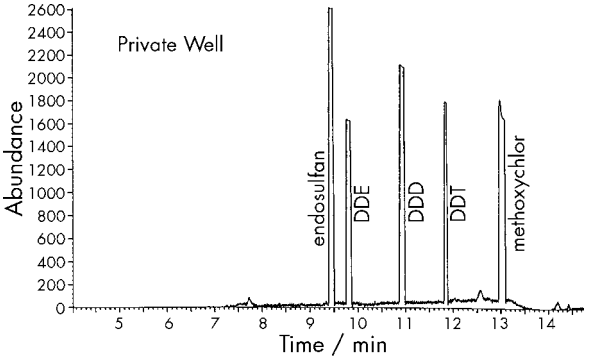


Figure 3. Representative GC of the private well water source.

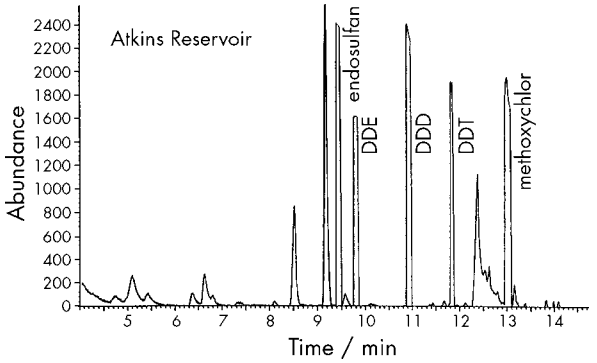


Figure 4. Representative GC of the Atkins Reservoir water source.

and *m/z* 318 (loss of HCl), a moderate peak at *m/z* 246 (loss of Cl_3), and a strong peak at *m/z* 235 (loss of CCl_3). The mass spectral fragmentation patterns of five pesticides are shown in Table 2.

By comparing the integrated area for each pesticide peak in their chromatograms with standards, the students determined the concentration of the pesticide in the ethyl acetate and calculated the concentration of the sampled pesticides in the drinking water they collected. Class averages for the pesticide concentrations for each of the three sampling sites are shown in Table 3.

Nuezil and Stone report that this method extracts 81% of the DDE in standard assays (*6*). In all cases, the levels of pesticides in the drinking water were below 1 ppb ($\mu g\ L^{-1}$).

Analysis of Pesticides by Enzyme-Linked Immunosorbent Assays of Cyclodiene (Endosulfan) and DDT

Antibodies to the DDT family and to pesticides containing cyclodienes (endosulfan) have been isolated from mice and bound to the sides of test tubes in commercially available ELISA kits. Students were introduced in prelab to the basic theory behind the ELISA (enzyme linked immunosorbent assay). They learned that the assay is based on the tight and essentially irreversible binding of the pesticide to antibody molecules that are covalently attached to the sides of plastic tubes into which the water samples are placed. Since these assays are very sensitive (sensitivity 1–10 ppb reported in product literature) and specific to a class of molecules, detection of minute quantities of one kind of pesticide in samples containing other contaminants is possible. The reporter molecule is an enzyme catalyst that can cleave a bond in a colorless substrate molecule and convert it into a yellow product molecule. This enzyme is conjugated with a similar pesticide molecule that will also bind to the antibody *if* the antibody has not already bound a pesticide molecule in the drinking water sample. Therefore, the deeper the yellow color in the product tube, the greater the amount of enzyme that has bound to the antibody and by extension, the smaller the amount of pesticide present in the original sample.

Though the technology is relatively sophisticated, the experimental procedures are quite simple, since this is a kit designed for amateurs to use in the field. Details are provided below for the cyclodiene assay; the DDT assay is similarly straightforward (we used the DDT soil analysis kit as directed) and details are provided with the kit.

For the cyclodiene assay, 0.160 mL of the drinking water sample was placed in a special test tube coated with antibodies to cyclodiene-containing pesticides. Four drops of a second solution containing an enzyme–pesticide conjugate were added and the solution was swirled for several seconds. After 5 min, the conjugate solution was discarded and the tubes were washed three times with deionized distilled water. Four drops of a solution containing a colorless enzyme substrate were added, plus four drops of developer. After 3 min the conversion of this colorless substrate into a colored product was "stopped" by the addition of one drop of 2.5 N sulfuric acid. With the addition of 0.5 mL of deionized distilled water, the sample was ready for analysis.

Table 2. Mass Spectral Fragmentation Patterns of Pesticides

Compound	*m/z* Peaks	Relative Intensity	Origin
DDT	354	14	Parent
	235	100	CCl_3 loss
	165	48	Cl_2 loss
DDD	320	20	Parent
	235	100	$CHCl_2$ loss
	165	52	Cl_2 loss
DDE	318	79	Parent
	246	100	Cl_2 loss
	176	56	C_6H_4 loss
Methoxychlor	344	7	Parent
	227	100	CCl_3 loss
Endosulfan	339	31	SO_2 loss
	241	91	CCl_2 loss
	195	100	C_2H_4Cl loss

The absorbance of the sample at 450 nm can be used to quantify the concentration of pesticide present using absorption spectroscopy in the visible region. Standard curves were prepared ahead of time and provided to the students and the students were shown how to convert an absorbance reading into a concentration. For the cyclodiene test, there are, in fact, several pesticides that will give a positive response, including endosulfan, aldrin, chlordane, and heptachlor. This test cannot distinguish between the compounds and yields only the *total* concentration of cyclodienes. Similarly, the DDT ELISA detects the total amount of DDT, DDD, and DDE. In most cases, the concentrations of pesticides in our test samples were low enough (below 1 ppb for cyclodienes and below 0.2 ppb for DDT) to give deep yellow colors and high absorbances, which signal levels of pesticides below the detection limit for the ELISA.

Because of the cost of the assay ($10/test) the class was divided in half; six groups tested for DDT and the other six groups tested for endosulfan. This way, each site had at least two independent ELISA measurements per day.

Molecular Modeling of Pesticides

Using basic ideas introduced in class on molecular shape as predicted for small molecules from simple theories of the valence shell electron pair repulsion (VSEPR) model, students

Table 3. Experimental Results of Water Source Analysis

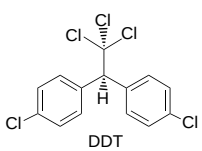
Compound	Standard Mix		Atkins Reservoir		Lawrence Swamp		Private Well	
	Peak Area	Concn/ppb	Peak Area	Concn/ppb	Peak Area	Concn/ppb	Peak Area	Concn/ppb
Endosulfan	1.27×10^6	1.001	1.36×10^5	0.157± 0.002	1.43×10^5	0.164± 0.013	1.38×10^5	0.159± 0.008
DDE	1.32×10^6	0.999	8.85×10^4	0.084± 0.006	8.71×10^4	0.082± 0.003	8.54×10^4	0.081± 0.001
DDD	1.52×10^6	0.986	1.21×10^5	0.101± 0.006	1.21×10^5	0.101± 0.006	1.20×10^5	0.100± 0.005
DDT	9.05×10^5	1.000	6.63×10^4	0.616± 0.025	6.49×10^4	0.603± 0.043	6.41×10^4	0.596± 0.043
Methoxychlor	1.51×10^6	1.000	1.50×10^5	0.116± 0.005	1.46×10^5	0.113± 0.008	1.39×10^5	0.108± 0.006

were asked to predict the shapes of the much more complicated pesticide molecules through a series of questions that had them consider individual parts of the molecules one at a time. The students progressed from a detailed consideration of the shape of DDT (2,2-bis(4-chlorophenyl)-1,1,1 trichloroethane), using both simple VSEPR theories and the modeling program Hyperchem, through the DDT metabolites DDD (2,2-bis(4-chlorophenyl)-1,1dichloroethane) and DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene. They were first asked to make predictions about bond angles on the basis of VSEPR and hybridization models and then to check their predictions on structures that had been calculated using more sophisticated theories. When differences were observed, the students were asked to propose reasons why the simple models may have failed them. This allowed for a discussion of ring distortion and resonance.

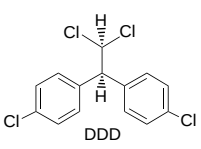
Students were introduced to the idea of torsion angles and taught how to measure these using Hyperchem. The DDT and related structures are useful examples of molecules for which single bond rotation (that about the carbon bridging the two chorophenyl groups) can produce different structures with different energies. Students then created a structure whose energy was not minimized and compared it to one whose energy had been minimized. In a final analysis, they were asked to think about the entire molecular structure using both ball-and-stick and space-filling models. They considered which parts of the molecule are flat, which are bent, and which might be considered hydrophobic or electronegative. In a stepwise progression, they did similar analyses on methoxychlor, endosulfan, and estrogen. Finally, we introduced them to the basis of hormone–receptor interactions, using the simple concept of a receptor protein acting as a lock and the estrogen hormone molecule acting as a key, and left them with the question of whether there is enough similarity in the structures of these pesticides and the structure of estrogen to support the proposal that they all bind to the same receptor protein. The theoretically modeled 3-dimensional structure of the estrogen receptor complex (pdb structure 1AKF, Maalouf et al. [*7*]) is now available for the students to visualize the "lock" and "key" in this context.

Molecular Structures of the DDT Family

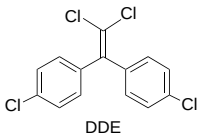
Shown below are the molecular structures of the family of DDT molecules. The students made predictions about bond angles using VSEPR from these modeled structures, and then built these molecules using Hyperchem and compared their predicted bond angles with those in the computer model. Finally, they considered the dihedral angles about the bridging chiral carbon and compared their unrefined structures with structures minimized using the Hyperchem energy minimization program.



DDT



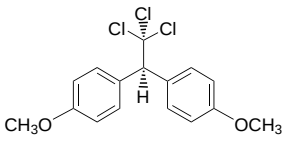
DDD



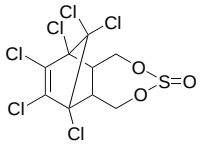
DDE

Molecular Structures of Methoxychlor and Endosulfan

Shown below are the structures for methoxychlor and endosulfan. Students were not asked to build these structures, but were given the computed minimized structures calculated as noted above. The structure of methoxychlor can be easily compared to the structure of the DDT family. The endosulfan structure was presented and the students were asked to observe that this structure is markedly different from all of the other structures. In an optional exercise for the more motivated students, this more complicated structure afforded the opportunity to discuss the 3-dimensional structures of saturated 6-membered rings (chairs and boats), the difference between axial and equatorial positions for peripheral atoms, and heteroatom influence in structure.



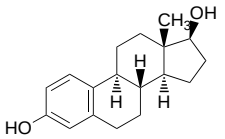
Methoxychlor



Endosulfan

The Molecular Structure of Estrogen

Shown below is the molecular structure of the estradiol molecule whose biological function is hypothesized to be perturbed by the xenoestrogenic pesticides. Students were asked to determine which pieces of the structure are like that of the DDT-derived molecules and which pieces are different. Finally, they critiqued the hypothesis that the xenoestrogenic pesticides work by binding directly to the estrogen receptor.



Estradiol

Conclusions

The write-up for the first phase of this experiment included an introduction stating the goal of the lab and an experimental section in which the ELISA and GC–MS procedures were described in general terms. Any deviations the students may have made from the stated instructions were noted. The data section included the location of the sampling site, the pH and temperature of the initial sample, the results for the GC–MS standard, the output from the GC–MS, the standard curves for the ELISA, and ELISA results for the site. The concentrations in both ppb and ppm and the molarity were calculated for the individual DDT, DDD, DDE, methoxychlor, and endosulfan peaks from the GC–MS. The students were asked to comment on the precision and accuracy of the numbers, as well on as the reliability of their own individual numbers. The individual groups were asked to combine their results to generate a class report.

Molecular modeling using Hyperchem allowed students to see for themselves how molecules can adopt 3-dimensional conformations and how those conformations can have different energies. By building a simpler structure such as DDT using molecular models (ball-and-stick) and then comparing the structures they had built with a minimized-energy structure,

students gained an appreciation for the flexibility of molecules. By comparing the overall 3-dimensional shapes of the pesticides with each other and with estrogen, students observed that it is not reasonable to accept a hypothesis that xenoestrogenic pesticides have a 3-dimensional structure similar to that of estrogen. Though one might argue that there are elements of similarity, it is clear that these pesticides do not "look" like estrogen. The write-up for the experiment was done in the laboratory, providing an environment for questions and discussion.

We plan to offer the course again with a few modifications. First, we have linked with a sixth-grade class and a high school chemistry class in Amherst's public school system. The three classes will work together to broaden the project to include sampling household drinking water, initial characterization of pH and conductivity, and spreadsheet management by the sixth graders, solid-phase extraction of samples by the high school students, demonstrations of ELISA and GC–MS for them by our college students, and final GC–MS, ELISA, and data analysis by our college students. We will have groups reporting to one another, and the group as a whole will report to the town water commissioner. Students can opt to make an oral presentation on some aspect of the program to either the high school or sixth-grade class in addition to the formal write-up for weeks I, II or III.

Acknowledgments

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Notes

1. It is important that the SPE columns be dried as thoroughly as possible before extraction because incompletely dried columns will produce a two-phase solution that complicates the analysis.
2. Samples eluted in ethyl acetate are not stable over time, so we advise that samples be analyzed the day they are extracted.
3. All solvents were ultrapure to prevent contamination.

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Additional Reading

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Interesting and Helpful Web Site Addresses (accessed Oct 1999)

EPA Methods

http://www.epa.gov/OWOW
http://www.ewg.org

Molecular Structure and Properties of Xenoestrogens

http://website.lineone.net/~mwarhurst/
http://cmit.keene.edu/faculty/rblatchly/Estogenics/EstrogenicCentral.html

Relative Risks of Pesticides

http://www.atsdr.cdc.gov/mrls.html