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Alteration of Mammalian-Cell Toxicity of Pesticides by Structural Iron(II) in Ferruginous Smectite

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The ultimate concern over pesticides in the environment is their toxic impact on nontarget organisms, including humans. Soil clays are known to interact with pesticides in ways that decrease the concentration of the parent compound in the soil solution (adsorption, sequestration, degradation). These phenomena are generally regarded as beneficial, but toxicological verification is lacking. In this study, mammalian-cell cytotoxicity of four commonly used agricultural chemicals (2,4-D, alachlor, dicamba, and oxamyl) was assessed after exposure to either reduced or oxidized ferruginous smectite (SWa-1). Results revealed that treatment with reduced smectite produced differential effects on mammalian-cell viability, depending on the pesticide. Oxamyl and alachlor reacted with reduced SWa-1 showed a significant decrease in their overall cytotoxic potential. Dicamba reacted with the reduced-clay treatment and generated products that were more toxic than the parent pesticide. Finally, no differences were observed between redox treatments for 2,4-D. The significance of these results is that oxidized smectites have virtually no influence on the toxicity of pesticides, whereas reduced-Fe smectite plays an important role in altering the cytotoxic potential of agricultural pesticides. The Fe oxidation state of clay minerals should, therefore, be taken into account in pesticide management programs.

Introduction

Each year, an estimated 4.5 billion pounds (2.04×10^9 kg) of a broad range of pesticides are consumed in the United States (*1*). Current use of conventional agricultural and commercial pesticides in the United States is estimated at about 1.23 billion pounds (5.58×10^8 kg) annually (*1, 2*), with expenditures by the agricultural sector at approximately $8.3 billion (*1*). While many economic benefits accrue from agricultural chemicals, they pose risks associated with their toxicologic impact on biological systems, including humans, and the environment (*3*−*5*).

Agricultural pesticides are primarily applied directly to the soil or indirectly through postemergence methods (*6*). Most detoxification processes of pesticides in the soil are coupled with degradation (*7*), so the ability of the soil to degrade these pesticides affects their toxic and environmental impacts. While abiotic processes of degradation were suggested for some pesticides, soil microorganisms have long been considered the principal pathway for pesticide degradation (*3, 6, 8, 9*). The mineral component of soils, especially clay minerals, is generally regarded as a catalyst for enhancing or inhibiting the availability of organic material to the microorganisms for biodegradation (*6, 10*−*13*).

The adsorption and sequestering of pesticides may also play a significant part in affecting the risks they pose to organism health. Soil organic matter is normally believed to dominate the adsorption of these compounds in the environment (*10, 11, 14, 15*), but evidence suggests that the effect of clay minerals is also significant (*14*). The adsorption of atrazine and alachlor on both organic and inorganic soil constituents has been widely studied (*16, 17*) and is well correlated with organic matter and clay content. Soil pH, temperature, moisture content, exchangeable cations, and electrolyte concentrations also affect the adsorption and degradation of herbicides (*18*−*22*).

Many studies reported that reduction of structural Fe(III) to Fe(II) in the crystal structure of pure clay minerals significantly changes the chemical fate of surface species (*23*−*31*). The effect of oxidation state of structural Fe in model clay minerals on the adsorption and degradation of pesticides in the environment is rather dramatic. Atrazine, alachlor, trifluralin, oxamyl, and chloropicrin have specifically been investigated (*27*−*31*). As compared to oxidized clays, reduction by either chemical or microbial treatments decreased the concentrations of these pesticides in the surrounding solution, by increased sorption and degradation. Reoxidized clay exhibited similar behavior toward the pesticide, as did the oxidized clay.

Do sorption and degradation, however, actually mitigate the adverse effects of pesticides on the environment and organism health? Despite the strong empirical evidence for profound effects of clay surface chemistry and structural Fe oxidation state on pesticide adsorption and degradation, no study was found regarding the impact of these effects on pesticide toxicity. Until this information is available and understood, prediction of the potential benefit of soil clays and their redox modification against the adverse toxic effects of agricultural chemicals is impossible.

The objective of this study was to characterize the effects of Fe oxidation state in smectite clay minerals on mammalian-cell cytotoxic potential of four commonly used agricultural chemicals (2,4-D, alachlor, dicamba, and oxamyl). Our central hypothesis was that pesticides reacted with reduced ferruginous smectite (SWa-1) clay will be less cytotoxic to mammalian cells than the unreduced or oxidized counterparts of the smectite. A three-fold approach to test this hypothesis was employed. First, a chronic mammalian-cell cytotoxicity assay was used to assess cytotoxic response of each supernatant from each treatment. Second, a tetrazolium dye sodium (XTT) assay was used as a secondary approach to cytotoxic response, in conjunction with cellular metabolic fitness. Finally, high-performance liquid chromatography (HPLC) analysis was used to confirm chemical correlation with the observed biological responses.

Materials and Methods

Chemicals. Reagent-grade or better of 2,4-dichlorophenoxy acetic acid (2,4-D); 2-chloro-2′,6′-diethyl-*N*-(methoxymethyl) acetanilide (alachlor); 3,6-dichloro-*O*-anisic acid (dicamba); *N*,*N*-dimethyl-2-methylcarbamoyloxyimino-2-(methylthio)-acetamide (oxamyl); ethyl methanesulfonate (EMS); and

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dimethyl sulfoxide (DMSO) were used. All pesticides were purchased from ChemService, Inc, West Chester, PA. The tetrazolium dye sodium (2,3-bis(2-methoxy-4-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) and Menadione used in the XTT assay were purchased from Sigma Chemical Co., St. Louis, MO. Media supplies and fetal bovine serum (FBS) were purchased from Hyclone Laboratories, Logan, UT. Sodium dithionite ($Na_2S_2O_4$) (purified grade, Fisher Chemical, Fair Lawn, NY), C-B buffer (consisting of sodium citrate (0.6 M) and sodium bicarbonate (2 M) mixed 8:1 by volume), and sodium sulfate (Na_2SO_4) (certified grade, Fisher chemical) were the reagents used for clay reduction treatments. Acidic H_2O and acetonitrile (Fisher Chemical, Fair Lawn, NY) were used as HPLC mobile phase. General laboratory reagents and supplies were purchased from Fisher Scientific Co. (Itasca, IL) and Sigma Chemical Co. (St. Louis, MO). All water used was doubly distilled and deionized (18 MΩ/cm) (DD H_2O).

Clay Minerals. The Na-saturated, <2-μm size fraction of ferruginous smectite (sample SWa-1 from the Source Clays Repository of The Clay Minerals Society) from Grant County, Washington, was used in this study. The clay was fractionated, dialyzed, freeze-dried, and redispersed at a concentration of 26.2 mg/mL prior to use (*32*). A 7.5 mg/mL stock suspension was prepared from the 26.2 mg/mL suspension.

Chemical Reduction. Reduced and oxidized smectites (SWa-1) were prepared from the unaltered clay as described by Stucki et al. (*32*). Excess solutes were removed from the clay by centrifuge washing three times with degassed 5 mM NaCl under inert atmosphere conditions. After the final decanting, the oxidized and reduced samples were resuspended at a concentration of 5 mg/mL.

Cells and Media. Chinese hamster ovary (CHO) cells, line AS52, clone 11-4-8, were obtained from Dr. Elizabeth Wagner (*33*). Cells were maintained in Ham's F12 medium with 5% FBS and grown at 37EC and 5% CO_2.

Pesticide Transformation by Reduced-Fe Smectite. Methods used for pesticide transformation by reduced-Fe clay were based on methods described by Tor et al. (*29*) and Xu et al. (*30*). Pesticide concentrations were selected on the basis of previously established concentration curves (*34*).

Alachlor Interaction with Chemically Reduced Clay. One 3.6-mL aliquot of alachlor (378.8 μM) was added to three separate 5-mL Teflon tubes containing either 1.4 mL of reduced smectite (final concentration of 2.5 mg/mL), 1.4 mL of oxidized smectite (2.5 mg/mL), or 1.4 mL of DD H_2O, respectively. The final volume of each tube was 5 mL. Reaction with reduced smectite was under inert atmosphere conditions (*32*). All samples were placed on a rocker platform and allowed to mix at room temperature for 72 h.

Dicamba, Oxamyl, and 2,4-D Interaction with Chemically Reduced Clay. One 2-mL aliquot of 2,4-D (4 M), dicamba (8 M), or oxamyl (400 μM) was added to three separate 5-mL Teflon tubes containing 2 mL of 5 mg/mL reduced smectite, 2 mL of 5 mg/mL oxidized smectite, or 2 mL of DDH_2O, respectively. The final reaction volume in each tube was 4 mL. The reduced reaction mixture was handled under inert-atmosphere conditions using a glovebox (*32*). Based on kinetic measurements to determine equilibration time, the samples containing 2,4-D or dicamba were placed on a rocker platform and allowed to mix at room temperature for 24 h and the oxamyl samples were reacted for 4 h.

Treatment Solution Final Preparation. Following the specified reaction times, the reduced samples were removed from the rocker platform, and 300 μL (1.5 mM) of deoxygenated $CaCl_2$ solution was added to flocculate the clay. The supernatant fluid was immediately separated from the smectite by centrifugation (Sorvall RC 5c Plus; rotor SS34) at 17 542*g* for 20 min. After separation, the supernatant fluid was carefully removed from the tube using a 20-gauge stainless steel needle with a Teflon syringe (Hamilton Company, Reno, NV). The supernatant fluid was passed sequentially through sterile Teflon and sterile Nylon syringe filters (0.22-μm and 0.02-μm pore size, respectively). The filtrate was then passed through a sterile 18-gauge needle into a 5-mL, sterile glass vial and sealed with a screw cap. The sterile supernatant was then submitted to the toxicity studies or HPLC analysis.

High-Performance Liquid Chromatography. All pesticides were analyzed in aqueous solution by high-performance liquid chromatography using a Hewlett-Packard 1050 instrument with autosampler, pump, and ultraviolet (UV) detector attached to an integrator (Hewlett-Packard, model 3396B series 3028A20752). The column used was a PRP-1 reversed phase 5 μm × 4.6 mm × 250 mm (Hamilton Company, Reno, NV). The mobile phase for 2,4-D, dicamba, and oxamyl consisted of 60% acetonitrile + 40% acidic H_2O; and for alachlor, 95% acetonitrile + 5% acidic H_2O. The flow rate was 1 mL/min. The detector wavelength was 220 nm.

Chronic Microplate Mammalian-Cell Cytotoxicity Assay. The chronic microplate mammalian-cell cytotoxicity assay was used to analyze the impact of the supernatant from the pesticide-treated clays on the viability of CHO cells over a 72-h period. Methods employed were those described by Plewa et al. (*35*, *36*) and Sorensen et al. (*34*), which utilized a 96-well microplate in which cell density was measured by visible absorbance spectroscopy at 450 nm after live-cell fixation with crystal violet.

Mammalian-Cell XTT Microplate Assay with Concurrent Chronic Cytotoxicity Analysis. The mammalian-cell XTT microplate assay was used to measure population metabolic activity (*37*). This method is based on the metabolic reduction of XTT by various mitochondrial dehydrogenases to produce formazan, which has an absorbance maximum at 466 nm. Menadione is added to the system to serve as an electron coupling agent between the XTT and the mitochondria. Mitochondrial activity is directly related to metabolic fitness of the cell; so, the more XTT converted to formazan, the more fit the cell. A diminution in formazan production indicates, therefore, that metabolism has decreased, either due to cell death or due to a decrease in cell fitness. By comparing XTT results with those from the chronic microplate mammalian-cell cytotoxicity assay above, which measures only cell death, an estimation of the effects of the pesticide on cell fitness, short of death, can be obtained. The XTT stock solution was prepared aproximately 1 h before use as 2 mg/mL XTT in unsupplemented F12 medium and 1 μL of 10 mM menadione per 400 μL of medium. XTT was added to the medium and gently stirred and heated at approximately 62 °C for about 30 min, until the solution reached a clear, reddish-orange color, at which point the menadione was added. Menadione was reconstituted in 100% DMSO prior to being added to XTT solution.

Columns 1 and 2 of the 96-well microplate served as a blank and negative control, respectively. The wells for the remaining columns contained 3000 CHO cells (100 μL), 25 μL (4 × F12 + FBS), an increasing volume of supernatant fluid from each pesticide/clay interaction, to a maximum of 75 μL, and/or DD H_2O, to a total volume of 200 μL. Five replicate wells were prepared per increasing volume of supernatant fluid per reaction plate. Plates were covered with AluminaSeal and placed at 37 °C in 5% CO_2 for 69 h. Fifty microliters of the XTT solution containing 10 mM menadione was then added to 4 of the 8 negative and blank control wells and to all of the test wells. The microplate was returned to 37 °C in 5% CO_2 for 3 h. The total incubation time was 72 h, after which the microplate was shaken for 4 s to distribute the formazan product uniformly and then analyzed in a BioRad model 550 microplate reader at 450 nm. The microplate was removed from the microplate reader, the

TABLE 1. Cytotoxicity of Pesticides Treated with Ferruginous Smectite (SWa-1) As Gauged by $\%C_{1/2}$, the Concentration of Pesticide Which Suppresses Cell Growth by 50% As Compared to the Concurrent Negative Control[a]

pesticide	treatment	concn range tested (μM)	$\%C_{1/2}$ (μM)	r^2
2,4-D	pesticide alone	100−750	515.11	0.968
	oxidized SWa-1		608.43	0.911
	reduced SWa-1		609.67	0.927
alachlor	pesticide alone	9.47−142.1	27.93	0.999
	oxidized SWa-1		23.15	0.990
	reduced SWa-1		31.21	0.980
dicamba	pesticide alone	200−3000	3282.17[b]	0.922
	oxidized SWa-1		2860.23	0.933
	reduced SWa-1		2073.77	0.978
oxamyl	pesticide alone	10−150	83.13	0.984
	oxidized SWa-1		106.73	0.945
	reduced SWa-1		226.18[b]	0.861

[a] r^2 is the regression coefficient of the plot of cell growth versus initial pesticide concentration, from which $\%C_{1/2}$ was determined. [b] Extrapolated from the curve. The $\%C_{1/2}$ value for this treatment fell outside the concentration range tested.

wells were gently aspirated, and the microplate was prepared for cytotoxicity assay as described above.

Data Analysis. Analysis of both the cytotoxicity and the XTT data was performed in exactly the same fashion. The average absorbance of the blank column at 450 nm was subtracted from the absorbance of each well. The average absorbance of the negative control was set as 100%. The absorbance for each treatment-group well was then converted into a percentage of the negative control as a function of the pesticide concentration versus its concurrent negative control. Statistical analysis was performed on each plot to determine significant differences from its concurrent negative control and from other treatments. A two-factorial analysis of variance test was conducted, and if a significant F value of $P \leq 0.05$ was obtained, a Holm−Sidak multiple comparison analysis was conducted. In general, the power of the test statistic (β) was ≥ 0.8 at $\alpha = 0.05$. A positive response is based on a concentration response to treatment and significant differences between reduced clay versus no clay or reduced clay versus oxidized clay.

Results

Cytotoxicity. The objective of this research was to test the hypothesis that the oxidation state of structural Fe in smectite (sample SWa-1) affects the cytotoxicity of agricultural chemicals to mammalian cells. The concentration at which a dose response by the CHO cells was observed for the pesticide alone varied with each pesticide and determined the concentration range selected for each pesticide tested in this study. The corresponding concentration ranges for 2,4-D, alachlor, dicamba, and oxamyl (Table 1) were, respectively, 100− 750, 9.47−142.1, 200−3000, and 10−150 μM. A concentration response increase in toxicity to mammalian cells was observed for each pesticide solution tested, regardless of its pretreatment (no exposure to the clay [pesticide alone], exposure to oxidized clay, or exposure to reduced clay), but the degree of toxicity varied with pesticide and treatment (Figure 1, Table 1). In every case (with the exception of oxamyl at 150 μM), the cytotoxicity of pesticide solutions receiving the oxidized-clay treatment was statistically the same as that of the pesticide-alone treatment, indicating that exposing the pesticide to smectite in its oxidized state had no effect on pesticide cytotoxicity. For all pesticides studied except 2,4-D, however, a statistically significant difference was found in the treatment involving exposure of the pesticide to reduced-Fe smectite. A regression analysis of the data for each plot of supernatant response was generated, and a $\%C_{1/2}$ value was calculated (Table 1). The $\%C_{1/2}$ value is the concentration of the test agent that reduced the cell density by 50% as compared to concurrent negative controls (*35*, *36*).

A small, but measurable fraction of alachlor is degraded by reduced smectite (*30*) and was reflected in a corresponding change in cytotoxicity (Figure 1, Table 1). The reduction of Fe in SWa-1 decreased the CHO cell cytotoxicity of alachlor by a small, but statistically significant (two-factorial ANOVA test, $P < 0.05$ level) margin in the concentration range 9.47−47.4 μM. The corresponding $\%C_{1/2}$ values were 27.93[a], 23.15[a], and 31.21[b] μM, respectively, where like superscripts denote no statistical difference between the values and different superscripts denote statistically significant differences between the values.

The effects of Fe oxidation state on oxamyl were even more pronounced (Figure 1, Table 1). Values for $\%C_{1/2}$ were 83.13, 106.73, and 226.18, respectively, for the pesticide-alone, oxidized-clay, and reduced-clay treatments, where the superscripts have the same meaning as before. Cytotoxicity was, therefore, markedly decreased by the reduced-clay treatment as compared to the corresponding oxidized-clay and pesticide-alone treatments. Differences between treatments were statistically significant above 50 μM ($P < 0.01$). At the greatest pesticide concentration (150 μM), treatment with the oxidized clay created a product that was statistically less toxic than the pesticide alone, but still substantially more toxic than treatment with the reduced clay. Even with this difference noted, structural Fe(II) in the smectite had the greatest impact on decreasing the cytotoxicity of oxamyl (Figure 1, Table 1).

Unlike alachlor and oxamyl, treatment of dicamba with reduced SWa-1 actually increased its overall CHO-cell cytotoxicity (Figure 1, Table 1). The corresponding values for $\%C_{1/2}$ were 3282.17, 2860.23, and 2073.77 μM, respectively. The increased dicamba cytotoxicity due to reduced SWa-1 smectite occurred at concentrations above 1000 μM ($P < 0.01$) and represents a 160% increase. No statistically significant differences were observed between the oxidized-clay and pesticide-alone treatments. The structural Fe(II), once again, induced the greatest change, but this time created an increase rather than a decrease in the pesticide's cytotoxicity.

In contrast to results for the other three pesticides, reduced smectite had no effect on the toxicity of 2,4-D throughout the concentration range 100−750 μM (Figure 1, Table 1). No consistent or significant dose−response differences were observed, either between the reduced- and oxidized-clay treatments or between the oxidized-clay and pesticide-alone treatments.

XTT. The chronic mammalian-cell XTT microplate assay assesses cell metabolic fitness and was applied to cells exposed to four pesticides after oxidized-clay, reduced-clay, and no treatments. Regression analyses of XTT metabolism versus pesticide concentration were performed, and the corresponding $\%C_{1/2}$ values were calculated for each solution treatment (Table 2).

Results (Figure 2) revealed that all four pesticides greatly decreased the metabolic fitness of the CHO cells as compared to the corresponding concurrent negative controls. Loss of metabolic fitness is the result of cell death or the slowing of metabolic activity or both. These factors can be separated by comparing the overall trends of the curves for XTT versus concentration (Figure 2) with those for cytotoxicity versus concentration (Figure 1, which measures only cell death). If the trends are similar, the metabolic digestion of XTT can be attributed entirely to cell death, whereas deviations in the curve trends indicate also the loss of metabolic activity short of cell death. For 2,4-D, alachlor, and dicamba, the observed

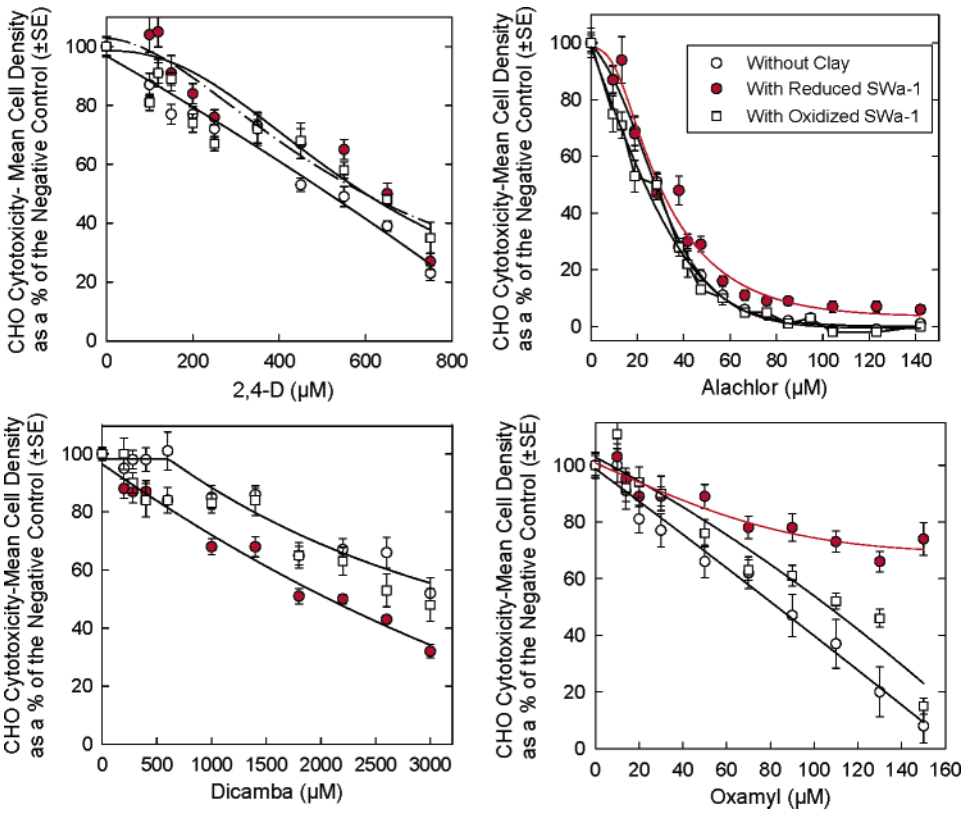

FIGURE 1. Cytotoxicity of pesticides 2,4-D, alachlor, dicamba, and oxamyl; comparing treatments with oxidized and reduced ferruginous smectite (SWa-1). Each concentration tested includes standard error bars (SE).

TABLE 2. XTT Analysis of Pesticides Treated with Ferruginous Smectite (SWa-1) As Gauged by $\%C_{1/2}$, the Concentration of Pesticide Which Decreases Cell Density by 50% As Compared to the Concurrent Negative Control[a]

pesticide	treatment	concn range tested (μM)	$\%C_{1/2}$ (μM)	r^2
2,4-D	pesticide alone	70−750	574.61	0.977
	oxidized SWa-1		591.57	0.993
	reduced SWa-1		615.15	0.971
alachlor	pesticide alone	13.3−142.10	44.26	0.999
	oxidized SWa-1		52.41	0.997
	reduced SWa-1		52.58	0.993
dicamba	pesticide alone	280−3000	1734.51	0.982
	oxidized SWa-1		2275.52	0.902
	reduced SWa-1		3174.61[b]	0.909
oxamyl	pesticide alone	14−150	40.26	0.999
	oxidized SWa-1		40.79	0.994
	reduced SWa-1		92.57	0.995

[a] r^2 is the linear regression coefficient of the plot of cell density versus initial pesticide concentration, from which $\%C_{1/2}$ was determined. [b] Extrapolated from the curve. The $\%C_{1/2}$ value for this treatment fell outside the concentration range tested.

trends reported in Figure 1 are clearly similar to those reported in Figure 2, so virtually all of the XTT digestion is attributable to cytotoxicity. For oxamyl, however, the curves change from linear or slightly curvilinear to S-shaped, indicating that oxamyl not only kills cells but also degrades the metabolic fitness of surviving cells.

Regarding the effects of the three pretreatments on the effect of the pesticide on metabolic fitness, statistical analyses (two-factorial ANOVA) found no statistical significance in the differences in $\%C_{1/2}$ values (Table 2) between pesticide-alone and oxidized-clay treatments of each pesticide, nor between the oxidized-clay and reduced-clay treatments, except in the case of dicamba at its highest concentration (3000 μM) and of oxamyl over the whole concentration range tested. For alachlor, the concentration range tested was 13.3−142.1 μM, and the $\%C_{1/2}$ values from the oxidized- and reduced-clay treatments were slightly greater than those from the no-clay treatment, yet these differences were not statistically significant ($P > 0.05$, Table 2). The concentration range tested for 2,4-D was 70−750 μM (Table 2), and no statistically significant differences were observed between the treatment groups (Figure 2, Table 2).

The $\%C_{1/2}$ value (92.57 μM) for oxamyl treated with reduced smectite was, however, significantly different from that of the pesticide-alone and oxidized-clay treatments in the concentration range of 40−150 μM, $P < 0.01$ (Table 2). This effect of reduced smectite follows the same pattern as observed in the cytotoxicity analysis (Table 1) and indicates that the metabolic fitness of cells exposed to oxamyl treated with reduced smectite was much greater than when oxamyl was treated with oxidized or no smectite. The cells also remained more metabolically active at greater initial concentrations of the pesticide when the oxamyl was first treated with reduced smectite.

Dicamba was the only other pesticide in which an effect of Fe oxidation state on XTT digestion was observed. The concentration range tested was between 280 and 3000 μM (Table 2), and a statistically significant difference between the pesticide-alone and oxidized-clay treatments or the reduced-clay treatment was observed only at the highest concentration, giving $\%C_{1/2}$ values of 1734.51, 2275.52, and 3174.61 μM.

Comparing results from the cytotoxicity (Table 1) and metabolic fitness (Table 2) tests revealed some differences in $\%C_{1/2}$ values between these two methods for assessing toxic effects. For example, in the case of dicamba, the value from the no-clay treatment decreased from 3282.17 μM in the cytotoxicity analysis to 1734.51 μM in the XTT analysis (Tables 1 and 2); yet, the trend between the two curves

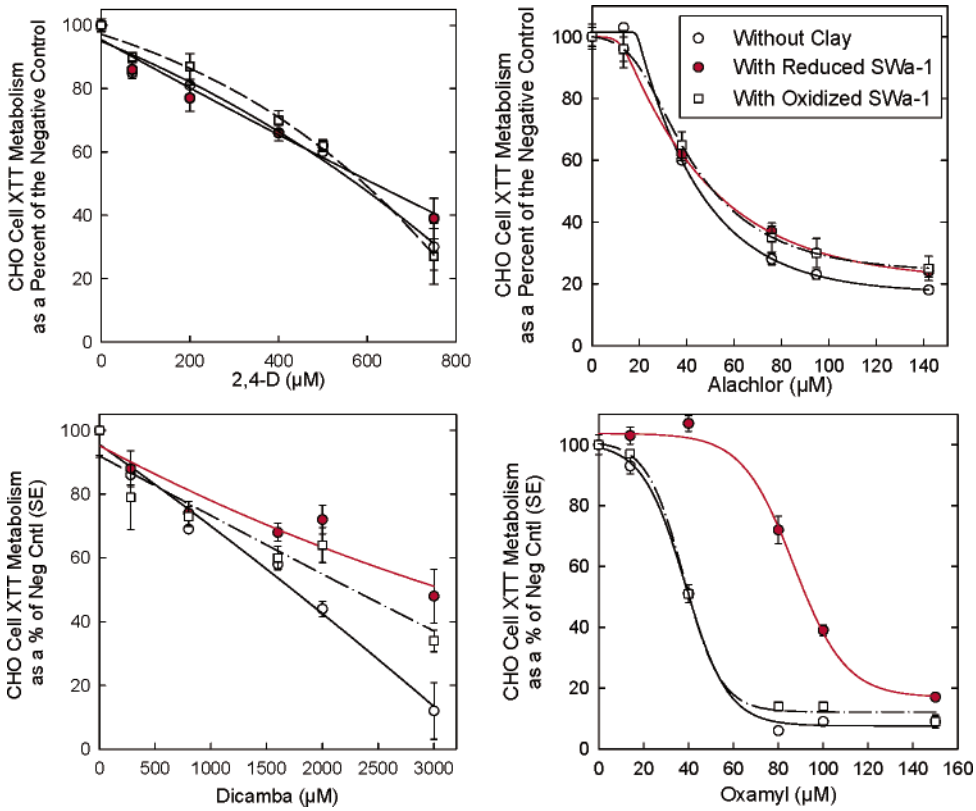

FIGURE 2. XTT analysis of pesticides 2,4-D, alachlor, dicamba, and oxamyl; comparing treatments with oxidized and reduced ferruginous smectite (SWa-1). Each concentration tested includes standard error bars (SE).

remained the same (Figures 1 and 2). The position of the curve for the reduced treatment changed from that of the cytotoxicity analysis (Figures 1 and 2), and the $\%C_{1/2}$ value for the reduced sample in the XTT analysis increased from 2073.77 μM observed in the cytotoxicity analysis to 3174.61 μM (Tables 1 and 2), but the trends of the curves are the same. Such differences obtained from the two approaches to toxicity measurements are understandable if the nature of each measurement is considered. Cytotoxicity measurements depend only on the presence of surviving cells, which are then stained with crystal violet, whereas in the XTT analysis a more complex array of reactions are involved—reduction of XTT, electron shuttle, and electron transfer within the cell—which leaves open the possibility for variations in efficiency and sensitivity.

HPLC Analysis. Results from HPLC analysis for alachlor and oxamyl (data not shown) were consistent with previous reports (*27*, *28*, *30*), revealing the appearance of secondary (degradation) products that correlated well with the disappearance of the parent compound (alachlor and oxamyl) upon treatment with the reduced clay. No such products were observed upon oxidized-clay treatment. These results not only confirm previous observations but, because concentrations used in previous studies were below those used here, extend the concentration range over which this phenomenon can be observed. Identification of these degradation products has yet to be accomplished.

HPLC analysis of dicamba found only the parent compound after the no-clay and oxidized-clay treatments, but a small (3%) secondary peak was observed after the reduced-clay treatment. While the identity of this product from dicamba is unknown, a possible candidate is 3,6-dichlorosalicylic acid (3,6-DCSA) (*40*). Previous studies of this compound (*40*−*43*) found it to adsorb rather strongly to soils and clay minerals, but no information is given regarding its toxicity. Regardless of identity, however, the degradation product evidently is highly toxic to CHO cells because, even in small quantity, it measurably enhanced cytotoxicity (Table 1, Figure 1).

For 2,4-D, the only peak observed after all three treatments was that of the undiminished parent compound. These results are consistent with the fact that clay treatment produced no change in the toxic behavior of 2,4-D toward CHO cells (Tables 1 and 2; Figures 1 and 2). No confirmation of these degradative behaviors of the smectite was possible from the literature because no information was found regarding the effects of Fe oxidation state in clay minerals on either dicamba or 2,4-D.

Discussion

In this study, the cytotoxicity to mammalian cells of four commonly used agricultural chemicals (alachlor, oxamyl, dicamba, and 2,4-D) was measured after exposure to both reduced and oxidized ferruginous smectite (SWa-1) clay. This is the first study to evaluate the effect of structural Fe oxidation state in a clay mineral on pesticide cytotoxicity to mammalian cells. Differences were observed in cytotoxic potency and metabolic fitness of CHO cells exposed to these pesticides treated with oxidized and reduced clay, as compared to a no-clay treatment. HPLC analysis confirmed that secondary products due to clay-dependent pesticide degradation occurred in three of the four pesticides. The de novo formation or an increase in percentage of secondary pesticide products coincided with increasing structural Fe(II) in the smectite. However, the effects of Fe oxidation state among pesticides tested were not uniform.

A significant and substantial decrease in CHO cytotoxicity of oxamyl treated with reduced SWa-1 was discovered; a slight, but statistically significant, decrease in cytotoxicity was observed for alachlor; an increase in CHO cytotoxicity was found for dicamba; and no differences were seen for 2,4-D as a function of the Fe oxidation state of smectite. The

lack of uniformity in results is not surprising in that variations of both degradation pathway and toxicologic impact for pesticides have been observed previously (*7*, *9*, *38*, *39*).

The finding that degradation of oxamyl and alachlor decreased CHO cell cytotoxicity agrees with the assumption that pesticide degradation is typically considered to be beneficial (*38*). The detection of an increase in cytotoxicity with dicamba after exposure to reduced smectite, however, is contrary to that hypothesis and raises the specter that benefits from clay−pesticide interactions greatly depend on the nature of the pesticide. In fact, the action of the clay could be completely neutral, evoking no response in the behavior of the pesticide as seen with 2,4-D. Such variability in the efficacy of this abiotic treatment of pesticides clearly must be considered when evaluating fate and impact of pesticides.

In past studies of clay−pesticide interactions, no efforts were made to establish or preserve a reduced-Fe state in the clay (*7*), so we assume that all such previous results compare best with the oxidized-clay treatments reported here, which showed little to no significant difference in the cytotoxic effects of the pesticides as compared to the pesticide without clay treatment. Sorption and sequestration of pesticides by clay minerals are typically regarded as principal consequences of clay−pesticide interactions, and these actions are environmentally beneficial (*6*, *16*, *17*, *30*, *38*). It is unknown whether pesticide sorption or sequestration occurred in the oxidized smectite of the present study, but results clearly indicate that if such sorption or sequestration did occur it was ineffective in altering the toxicity of the pesticide. In reduced smectite, the fact that toxicity increased in one case (dicamba) is evidence that the nature of the clay−pesticide interaction is degradation rather than sorption because the latter could only diminish, rather than add to, the cell exposure to pesticide. The beneficial effects of constituent clay minerals in the soil on mitigating adverse consequences of pesticide application may, therefore, be questionable, except under circumstances where changes occur in structural Fe oxidation state in the clay minerals.

The primary pathway for structural Fe reduction in clay minerals in natural soils and sediments is most likely via microbial reduction of Fe(III) to Fe(II) (*44*, *45*). Studies by Tor et al. (*29*) and Zhang et al. (*28*) illustrated that, while chemically reduced smectite had a greater effect on the degradation of pesticides, microbially reduced smectite also showed a significant effect. This raises the possibility that the work reported herein may, in fact, have application in the field where microbially reduced smectites can interact with pesticides and alter the environmental toxicity of the pesticide. This has implications for pesticide application efficacy and toxicity within wet soils (reducing conditions) and could be the basis for the use of redox processes as a remediation method for pesticide pollution.

While the present study was not designed to differentiate the relative effects of adsorption versus degradation of the pesticides on cytotoxicity, clay minerals are effective adsorption and sequestering agents for many pesticides (*8*, *9*, *21*, *46*); so, a discussion of the possible role of pesticide adsorption as a mechanism for decreasing cytotoxicity is warranted. HPLC data demonstrated that degradation products of oxamyl, dicamba, and alachlor are present after reduced-smectite treatment. Zhang et al. (*28*) reported minimal adsorption of oxamyl to smectite, so in this case degradation seems to be the clear mechanism for decreasing toxicity. Smith (*40*, *41*) observed minimal adsorption of dicamba to clay surfaces, but 3,6 DCSA is more adsorbent. If 3,6 DCSA is the secondary product produced from dicamba in the present study and if the assumption is correct that increased adsorption shields the organism from toxic activity of the compound, then we should have observed a decrease rather than an increase in toxicity after reduced-clay treatment of dicamba. Bosetto (*22*) demonstrated that alachlor has a relatively high adsorption potential to clay minerals, but we observed no change in toxicity of alachlor after reaction with oxidized smectite; and Xu et al. (*30*) found that adsorption of alachlor to reduced smectite surfaces is significantly greater than that to oxidized smectite, but we found that the diminution in cytotoxicity, even though statistically significant, is small. These observations suggest that adsorption may play only a minor or even insignificant role in modulating the toxicity of pesticides.

The increase in cytotoxic response induced by the reduced-clay treatment of dicamba indicates that pesticide degradation can be detrimental rather than beneficial. Secondary products from other pesticides are also known to have a greater toxic effect than the parent compound (*38*, *47*). The extent to which this type of degradative activation occurs over the wide array of available pesticides is, however, little known because toxicity analyses are vastly incomplete even of the parent compounds, let alone of the secondary products (*4*, *5*, *48*, *49*).

In this study, the chronic mammalian-cell cytotoxicity and XTT assays provided cost- and time-effective quantitative measurements of several pesticides and multiple treatments groups. Similarities in trends of $\%C_{1/2}$ values between these assays were noted; yet, the chronic mammalian-cell cytotoxicity assay appeared to be more sensitive in determining toxic impact. Comparisons between the two assays allowed for assessment of metabolic fitness of a cell population. The utilization of these types of tools in conjunction with standard soil chemistry and clay mineralogy may prove useful for assessing the toxicologic impact for proposed remediation mechanisms. In a previous study, we determined a relationship between $\%C_{1/2}$ values to known LD_{50} values of pesticides (*34*). These assays may serve as an initial analytical tool to assess the toxic impact of pesticides. In addition, while the effects of pesticide burden on microbial populations is of environmental importance, mammalian-cell toxicity has more direct implications for animal populations, including humans.

Finally, multiple degradation pathways and processes are typically suspected in heterogeneous soil and sediment samples (*3*, *7*, *9*). Previous studies have shown that the manipulation of Fe oxidation state in clay minerals can greatly affect the degradation and sorption of pesticides (*27*−*31*), but the toxicologic significance of these findings was not measured. The data presented here demonstrate that redox processes and the resulting changes in Fe oxidation state in clays can greatly impact the overall cytotoxic potential of agricultural chemicals. The redox state of soil clay minerals to which pesticides are exposed clearly must be considered when investigating the environmental fate and health risks of agricultural chemicals.

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Literature Cited

(1) U.S. EPA. *Pesticides Industry Sales and Usage 1996 and 1997 Market Estimates*; U.S. EPA: Washington, DC, 1997.

(2) USGS. *Pesticides in Stream Sediment and Aquatic Biota*; USGS: Washington, DC, 2000.
(3) van der Werf, H. M. G. *Agric., Ecosyst. Environ.* **1996**, *60*, 81−96.
(4) Thornton, J. *Int. J. Occup. Environ. Health* **2000**, *6*, 318−330.
(5) Rodricks, J. V.; Rachman, N. J. *Am. J. Ind. Med.* **1990**, *18*, 167−175.
(6) Moorman, T. B. In *Soil Biology: Effects on Soil Quality*; Hatfield, J. L., Stewart, B. A., Eds.; CRC Press Inc.: Boca Raton, FL, 1994.
(7) Sims, G. K.; Cupples, A. M. *Factors Controlling Degradation of Pesticides in Soil*; IUPAC Conference, 1999.
(8) Racke, K. D. *ACS Symposium Series*; American Chemical Society: Washington, DC, 1990; pp 1−12.
(9) Walker, A. *Aspects Appl. Biol.* **1989**, *21*, 159−172.
(10) Guo, L.; Bicki, T. J.; Felsot, A. S.; Hinesly, T. D. J. *Environ. Sci. Health* **1991**, *B26*, 513−528.
(11) Guo, L.; Bicki, T. J.; Felsot, A. S.; Hinesly, T. D. *J. Environ. Qual.* **1993**, *22*, 186−194.
(12) Hance, R. J. *Aust. Weeds* **1984**, *3*, 26−34.
(13) Yaron, B.; Gertsl, Z.; Spencer, W. F. *Adv. Soil Sci.* **1985**, *3*, 121−211.
(14) Laird, D. A. In *Herbicide Metabolites in Surface Water and Groundwater*; Meyer, M. T., Thurman, E. M., Eds.; American Chemical Society: Washington, DC, 1996; pp 87−100.
(15) Khan, S. U. *Pestic. Sci.* **1978**, *9*, 39−43.
(16) Dunigan, E. P.; McIntosh, T. H. *Weed Sci.* **1971**, *19*, 279−282.
(17) Laird, D. A.; Yen, P. Y.; Koskinen, W. C.; Steinheimer, T. R.; Dowdy, R. H. *Environ. Sci. Technol.* **1994**, *28*, 1054−1061.
(18) Ballard, J. L.; Santelmann, P. W. *Proc. South Weed Sci. Soc.* **1973**, *26*, 385−388.
(19) Dao, T. H.; Lavy, T. L. *Weed Sci.* **1978**, *26*, 303−308.
(20) Peter, C. J.; Weber, J. B. *Weed Sci.* **1985**, *33*, 874−881.
(21) Weber, J. B.; Peter, C. J. *Weed Sci.* **1982**, *30*, 14−20.
(22) Bosetto, M.; Arfaioli, P.; Fusi, P. *Soil Sci.* **1993**, *155*, 105−113.
(23) Cervini-Silva, J.; Wu, J.; Larson, R. A.; Stucki, J. W. *Environ. Sci. Technol.* **2000**, *34*, 915−917.
(24) Cervini-Silva, J.; Wu, J.; Stucki, J. W.; Larson, R. A. *Clays Clay Miner.* **2000**, *48*, 132−138.
(25) Yan, L.; Stucki, J. W. *Langmuir* **1999**, *15*, 4648−4657.
(26) Yan, L.; Stucki, J. W. *J. Colloid Interface Sci.* **2000**, *225*, 429−439.
(27) Zhang, L. M.S. Thesis, University of Illinois, Urbana−Champaign, IL, 2002.
(28) Zhang, L.; Larson, R. A.; Wu, J.; Kostka, J. E.; Stucki, J. W. *Clays Clay Miner.* (submitted for publication).
(29) Tor, J. M.; Xu, J. C.; Stucki, J. W.; Wander, M. M.; Sims, G. K. *Environ. Sci. Technol.* **2000**, *34*, 3148−3152.
(30) Xu, J. C.; Stucki, J. W.; Wu, J.; Kostka, J. E.; Sims, G. K. *Environ. Chem. Toxicol.* **2001**, *20*, 2717−2724.
(31) Kocherginsky, N. M.; Stucki, J. W. *Environ. Sci. Technol.* **2000**, *34*, 3574−3578.
(32) Stucki, J. W.; Golden, D. C.; Roth, C. B. *Clays Clay Miner.* **1984**, *32*, 191−197.
(33) Wagner, E. D.; Rayburn, A. L.; Aderson, D.; Plewa, M. J. *Environ. Mol. Mutagen.* **1998**, *32*, 360−368.
(34) Sorensen, K. C.; Stucki, J. W.; Plewa, M. J. *Bull. Environ. Contam. Toxicol.* **2003**, *70*, 1083−1088.
(35) Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D. *Water Sci. Technol.* **2000**, *42*, 109−116.
(36) Plewa, M. J.; Kargalioglu, Y.; Vankerk, D.; Minear, R. A.; Wagner, E. D. *Environ. Mol. Mutagen.* **2002**, *40*, 134−142.
(37) Woods, E. M.S. Thesis, University of Illinois, Urbana−Champaign, IL, 2002.
(38) Seiber, J. N. In *Handbook of Pesticide Toxicology Principles*; Krieger, R., Ed.; Academic Press: San Diego, CA, 2001; pp 643−648.
(39) Petersen, B.; Tomerlin, J. R.; Barraj, L. *Food Technol.* **1996**, 221−223.
(40) Smith, A. E. *J. Agric. Food Chem.* **1974**, *22*, 221−225.
(41) Smith, A. *J. Agric. Food Chem.* **1973**, *21*, 708−710.
(42) SERA. *Vanquish Risk Assessment*; United States Department of Agriculture: Washington, DC, 1994.
(43) SERA. *Vanquish Chemical Background Statement*; United States Department of Agriculture: Washington, DC, 1994.
(44) Stucki, J. W.; Komadel, P.; Wilkinson, H. T. *Soil Sci. Soc. Am. J.* **1987**, *51*, 1663−1665.
(45) Wu, J.; Roth, C. B.; Low, P. F. *Soil Sci. Soc. Am. J.* **1988**, *52*, 295−296.
(46) Mingelgrin, U.; Gerstl, Z. *J. Environ. Qual.* **1983**, *12*, 1−11.
(47) Coats, J. R. In *Pesticide Transformation Products: Fate and Significance in the Environment*; Somasundaram, L., Coats, J. R., Eds.; ACS Symposium Series 459; American Chemical Society: Washington, DC, 1991; pp 10−31.
(48) NRC. *Toxicity Testing: Strategies to Determine Needs and Priorities*; National Academic Science Press: Washington, DC, 1984.
(49) NRC. *The Future Role of Pesticides in U.S. Agriculture*; National Academic Science Press: Washington, DC, 2000.

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