Geochemical Modulation of Bioavailability and Toxicity of Nitroaromatic Compounds to Aquatic Plants

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Nitroaromatic compounds (NACs) are prominent soil and sediment contaminants that are strongly adsorbed by smectites at extents that depend on hydration properties of the exchangeable cation. Potassium smectites adsorb nitroaromatics much more strongly than calcium smectites, so that adjustment of K\(^+\) versus Ca\(^{2+}\) occupation on cation exchange sites in smectites can be used to modulate the retention and release of nitroaromatics. We suggest that this modulation can be used to advantageously manage the bioavailability and toxicity of NACs during bioremediation.

We have measured the toxicity of 2,4-dinitrotoluene (2,4-DNT) to duckweed grown in smectite suspensions and utilized Ca\(^{2+}/K^+\) exchange to retain or release 2,4-DNT. Retention by potassium smectite reduced bioavailability and hence toxicity to duckweed. Addition of Ca\(^{2+}\) to replace K\(^+\) by ion exchange released adsorbed 2,4-DNT, which is toxic to duckweed. So smectites can be used to sequester or release 2,4-DNT predictably and provide means to control bioavailability and environmental toxicity.

Introduction

Smectites are expandable 2:1 layered aluminosilicate clays that are widely distributed in nature (1). The basic clay sheet consists of an aluminum octahedral layer (Al\(^{3+}\) coordinated to eight oxygens) sandwiched between two silicon tetrahedral layers (Si\(^{4+}\) coordinated to four oxygens). Smectites have structural negative charges arising from isomorphous substitution (substitution of a lower valent cation for higher valent one) in the octahedral and/or tetrahedral layers. In their natural state, inorganic cations commonly found in nature associate with clay surfaces to balance the negative charges. These exchangeable cations strongly influence the interlayer environment of smectites via hydration. The exchangeable cations may interact with certain environmental contaminants and pesticides in the clay interlayers, leading to absorption from water and/or abiotic transformation. Among the clay minerals commonly found in soils, smectites often function as particularly effective adsorbents for organic contaminants and pesticides because of their high surface areas, large cation-exchange capacities (CEC), and reversible interlayer expansibility (2).

Sorption by soils and sediments is a major determinant of the environmental fate of organic contaminants and pesticides, including bioavailability to microorganisms and plants (3). Sorption often renders contaminants less available to receptor organisms (4–7). Steinberg et al. (8) and Scribner et al. (9) showed that ethylene dibromide and simazine sorption and sequestration by field-weathered soils reduced bioavailability to indigenous microorganisms capable of degrading these compounds, thereby prolonging residence times. Harris (10) showed lowered bioavailability (to crickets) of insecticides sorbed to dry mineral soils as evidenced by higher lethal concentrations (LC\(_{50}\)) compared to the insecticides in the same wetted soils. Apparently, the addition of water caused the release of adsorbed insecticides by soil mineral fraction, manifesting lower LC\(_{50}\) values. Soil minerals in the dry state have been shown to sorb higher amounts of certain organic contaminants and pesticides than that which occurs in the presence of water (11). It was hypothesized that water molecules were preferentially adsorbed on the mineral surfaces rendering the soil mineral fraction ineffective in the sorption of nonionic organic molecules. In contrast, certain important classes of pesticides and organic contaminants (e.g., nitroaromatics, triazines, dioxins) may be lost from water by clay minerals to a greater degree than by soil organic matter (12–14). For example, 2,4-dinitrotoluene (2,4-DNT) was highly competitive with water for adsorptive sites on potassium smectite. Sorption of aqueous phase 2,4-DNT by potassium smectite reduced its bioavailability and toxicity to the aquatic plant duckweed (15). A number of studies have examined the bioavailability of sorbed contaminants to microorganisms. Several studies provide evidence that bacteria can access contaminants sorbed to soils or clays (16–19). Thus, generalizations regarding the bioavailability of sorbed organics to plants and bacteria are not apropos.

Sorption of NACs by smectites is strongly influenced by the type of exchangeable cations occupying the clay, the clay CEC, and their respective hydration properties. Smectites exchanged with Cs\(^+\), K\(^+\), and NH\(_{4}^+\) have high affinities for NACs but display much lower NAC adsorption when exchanged with Na\(^+\), Ca\(^{2+}\), or Mg\(^{2+}\) (14, 20–25). Adsorption of NACs occurs primarily in the clay interlayers. We hypothesize that NAC mobility, bioavailability, and toxicity can be controlled by simple ion-exchange reactions on smectite clays. Li et al. (26) measured adsorption of dichlobenil by smectites exchanged with different ratios of K\(^+/-\)Ca\(^{2+}\) and found that adsorption increased with increasing molar ratio of K\(^+/-\)Ca\(^{2+}\) on the cation-exchange sites. We contend that this simple geochemical method for modulating retention and release of certain contaminants holds promise as a tool for the successful implementation of bioremediation technologies. For example, in the case of NACs at highly contaminated sites, their initial sequestration by potassium smectite might permit robust establishment of phytoremediative plants and microorganisms. Subsequent addition of Ca\(^{2+}\) could induce controllable release of NACs at nontoxic levels into an active rhizosphere, thereby optimizing the potential for successful biodegradation and/or plant uptake.

The objective of this study was to modulate the bioavailability and toxicity of 2,4-DNT by controlling its adsorption to, and release from, smectite via simple K\(^+/-\)Ca\(^{2+}\) exchange.
reactions on the mineral surfaces. The test organism used in this study was duckweed (*Lemna minor*), a group of free-floating aquatic plants commonly found growing on nutrient-rich water in ponds and lakes.

**Materials and Methods**

**Chemicals.** 2,4-Dinitrotoluene was purchased from Aldrich Chemical Co. (Milwaukee, WI) with a purity > 97%, KCl, CaCl₂, MgSO₄, KNO₃, KH₂PO₄, H₂BO₃, Na₂MoO₃, CH₃COONa, and CuSO₄ were obtained from Mallinckrodt Baker (Phillipsburg, NJ); FeCl₃, MnCl₂, and ZnSO₄ were obtained from Sigma (St. Louis, MO); and ethylenediaminetetraacetic acid (EDTA) was obtained from Invitrogen (Carlsbad, CA). These chemicals were reagent-grade and were used as received.

**Clay Preparation.** Smectite clay (Wyoming montmorillonite, SWy-2) was obtained from the Source Clays Repository of the Clay Minerals Society (Purdue University, West Lafayette, IN) and used throughout this study. This clay has a CEC of 82 cmol/kg and a theoretical surface area of 750 m²/g (27). The clay was first treated with 0.5 M sodium acetate to remove carbonate impurities (26). The ~2 μm particle fraction was obtained by centrifuging a dilute clay suspension for 6 min at 60g by use of a Sorvall RSA rotor and RC-5C centrifuge (DuPont, Wilmington, DE) (29). To prepare homoionic potassium smectite, exchangeable cations (predominantly Na⁺ due to prior treatment with sodium acetate) on SWy-2 smectite were replaced with K⁺ by washing the clay-sized fraction three times with 0.5 M KCl. Potassium smectite suspensions were then dialyzed against Milli-Q water (Barnstead/Thermolyne, Dubuque, IA) to remove excess electrolytes. The clay suspensions were quick-frozen in a solid CO₂/acetone slurry and freeze-dried. Prior to use in phytotoxicity assays, potassium smectite was sterilized by heating in an oven at 120 °C for 3 days. Sterility was verified by plating a sample of the sterilized clay slurry on Difco R2A agar (Becton Dickinson and Co., Sparks, MD).

**Duckweed Culture.** Duckweed was used as the indicator species for evaluating NAC exposure. Toxicity testing using duckweed is an accepted protocol for evaluating aquatic toxicities of organic and heavy metal contaminants (30–36). *Lemna minor* duckweed cultures were obtained from the Culture Collection of Algae and Cyanobacteria, University of Toronto, Toronto, Ontario, Canada. Axenic duckweed cultures were maintained in 300-ml Erlenmeyer flasks containing 150 mL of Hoagland’s nutrient solution (37) at pH 5.8, supplemented with 10g/L sucrose (Judy Acreman, personal communication, University of Toronto). After autoclave sterilization, 20 mL of a FeEDTA solution (prepared by dissolving 0.121 g of FeCl₃ and 0.375 g of EDTA into 250 mL of Milli-Q water and passing through a 0.22 μm filter) was added to complete the full-strength duckweed medium (1× DWM). Culture flasks inoculated with duckweed were closed with aluminum foil to reduce the evaporative loss of water. The effect of 2,4-DNT sorption by potassium smectite on duckweed growth was evaluated as follows. Prior to inoculation with duckweed, the flasks (four replicates for each measurement) were shaken for 4 h at room temperature. Then, 8–10 duckweed fronds were aseptically transferred to each flask, the foam plugs and foil were replaced, and the flasks were incubated in a growth chamber as described above. Thus, the final bioassays consisted of 75 mL of 0.2× DWM, 0.01 M KCl background electrolyte, 7.5 mg/L 2,4-DNT, 300 mg of potassium smectite, and 8–10 duckweed fronds. Controls were identical with clay but without 2,4-DNT. Total duckweed frond numbers were counted manually in each flask at selected sampling times up to 17 days of incubation as a measure of growth. At preselected sampling times of 0, 4, 9, 11, and 17 days, aliquots (2 mL) of solution were sampled, and the 2,4-DNT concentrations were measured by HPLC. The test concentration of 7.5 mg/L 2,4-DNT was selected because in aqueous solution this level was toxic to duckweed but could be lowered to a nontoxic level by adding a moderate amount (300 mg) of potassium smectite according to a dose–response curve reported previously (15).

**Release of 2,4-DNT into Solution.** After a 4-day initial incubation at which time 20 duckweed fronds were established, 75 mL of 0.1 M CaCl₂ in 0.2× DWM was added to induce the release of sorbed 2,4-DNT as Ca⁺⁺ replaced K⁺ on the exchange sites of potassium smectite. These flasks were gently shaken for 4 h and then incubated in a growth chamber for an additional 13 days. Controls were of the same composition but without either 2,4-DNT or potassium smectite, as well as those with the addition of 75 mL of 0.01 M KCl rather than 75 mL of 0.1 M CaCl₂.

A completely randomized design was adopted for data analysis. Before comparison of treatment differences, a natural log transformation was applied to the data because we observed larger variance at later time points than at earlier time points. The analysis of variance (ANOVA) was applied to determine the statistical significance (P < 0.05) of differences in frond numbers among treatments. Statistical analysis of data (PROC MIXED; SAS Institute, 2005) was performed to analyze treatment differences in duckweed frond numbers (38).

**Results and Discussion**

Smectite clay is an effective sorbent for 2,4-DNT, with the extent of sorption strongly dependent on the exchangeable cations occupying the exchange sites of the clay. Isotherms representing sorption of 2,4-DNT from 0.01 M KCl and 0.005 M CaCl₂ by potassium smectite are presented in Figure 1. Sorption of 2,4-DNT by potassium smectite was substantially...
However, an aqueous concentration of 0.01 M NH₄⁺ provide ancillary nutritional benefits for growth of duckweed. Lower adsorption from CaCl₂ solution is attributed to the exchange of some K⁺ from potassium smectite by Ca²⁺. This eliminates some K⁺-saturated domains in the clay interlayers where NAC sorption occurs (26). The favorable clay–NAC interactions afforded in the K⁺-saturated domains are diminished when Ca²⁺ occupies some fraction, but not necessarily all, of the exchange sites in such domains (26). The comparatively strong hydration of Ca²⁺ compared to K⁺ inhibits complex formation between the exchange ion and NACs, interactions with siloxane clay surfaces, and solute dehydration, which are all energetically favorable processes (14, 21–26). As a consequence, addition of Ca²⁺, and subsequent ion exchange of Ca²⁺ for K⁺ on smectite, induces the release of 2,4-DNT into solution.

For bioassays in the presence of clay, it was necessary to separate toxicity of 2,4-DNT from possible effects on growth associated with cations utilized in the cation-exchange process. The cations K⁺, NH₄⁺, and Ca²⁺ at aqueous concentrations between 0.01 and 0.2 M were tested for their effect on growth of Lemna duckweed (Figure 2). These cations are candidates for modulating the sorption and release of NACs by clays (26). Smectite clays saturated with K⁺ or NH₄⁺ are strong adsorbents for NACs, whereas calcium smectite is much less effective (Figure 1) (14). Ammonium might also provide ancillary nutritional benefits for growth of duckweed. However, an aqueous concentration of 0.01 M NH₄⁺ substantially reduced the number of fronds. At concentrations ≥ 0.05 M, NH₄⁺, K⁺, and Ca²⁺ all diminished the number of fronds produced. Since 0.01 M KCl had no negative effect on duckweed growth, it was chosen as the background electrolyte solution in our bioassays.

The duckweed L. minor is sensitive to the toxic effects of 2,4-DNT (15) and hence is an appropriate indicator species to evaluate the effect of Ca²⁺ – K⁺ exchange reactions on the bioavailability and toxicity of 2,4-DNT. Figure 3 shows the response of L. minor to the presence of 2,4-DNT in potassium smectite suspensions in terms of frond numbers. The 2,4-DNT aqueous concentration was also monitored during the 17-day incubation. At the initiation of the experiment potassium smectite was added, which substantially reduced the 2,4-DNT concentration in water from 7.5 to <0.3 mg/L, which had minimal impact on L. minor growth according to a dose–growth relationship published previously (14). This is also confirmed in the present study during the first 4 days of exposure, where there were no differences in duckweed growth (frond numbers) in the potassium smectite suspensions with added 2,4-DNT and the controls without 2,4-DNT (Figure 3A).

Since there were no significant differences in the number of fronds produced among treatments at day 4, a 0.1 M CaCl₂ solution was added as one treatment to initiate the ion-exchange process of Ca²⁺ for K⁺. Here we added Ca²⁺ at a concentration sufficient to release 2,4-DNT at levels that are toxic to duckweed, to demonstrate the ability to achieve Ca-induced release. In practice, this could be done gradually (e.g., with incremental additions at lower Ca²⁺ concentrations, Figure 1) to achieve release at nontoxic levels. L. minor growth curves were nearly identical for KCl-added systems whether 2,4-DNT was present or not (Figure 3A), showing that potassium smectite effectively sequesters 2,4-DNT and renders it unavailable. In the absence of 2,4-DNT, the addition of CaCl₂ reduced frond production about 3-fold compared to the treatment with KCl addition. In the suspensions containing 2,4-DNT sorbed by potassium smectite, the addition of CaCl₂ caused the aqueous concentration of 2,4-DNT to increase from <0.3 to 1.5 mg/L (Figure 3B). This aqueous concentration clearly inhibited duckweed growth. In the treatments where 0.1 M CaCl₂ was added to systems...
containing 2,4-DNT and potassium smectite, Ca\(^{2+}\) itself likely caused some inhibitory effects on duckweed growth, based on results shown in Figures 2a and 3A. However, the inhibitory effects of Ca\(^{2+}\) itself versus 2,4-DNT released into solution could be easily discerned (Figure 3A).

The Ca\(^{2+}\)-induced release of 2,4-DNT into solution (Figure 3B) caused near-complete cessation of L. minor growth. Comparisons were made among the treatments with 2,4-DNT and controls without 2,4-DNT at specific exposure times to monitor the temporal trends in 2,4-DNT toxicity to duckweed. The treatment effects were easy to discern. At day 4, just prior to when the bioassay flasks were amended with KCl or CaCl\(_2\), there were no significant differences in frond numbers among treatments. At day 7, in the KCl-amended systems, frond numbers with and without 2,4-DNT were not statistically different. In contrast, in treatments where 2,4-DNT was present, frond numbers in the CaCl\(_2\)-amended system were significantly lower than in the KCl + 2,4-DNT system. At subsequent times, differences between the CaCl\(_2\)+2,4-DNT and KCl + 2,4-DNT treatments remained significant with the later having higher frond numbers. By day 17, the difference in these two treatments was very large; 982 versus 76 fronds for the KCl + 2,4-DNT and CaCl\(_2\) + 2,4-DNT systems, respectively.

To further quantitate these effects, growth rates were calculated by fitting growth data (Figure 3A) to a first-order equation:

\[
\ln F_t = \ln F_0 + kt
\]

where \(F_t\) and \(F_0\) are frond numbers at time \(t\) (days) and initial time, respectively, and \(k\) (days\(^{-1}\)) is the growth rate constant. The results of fittings are shown as solid lines in Figure 3A. The growth data were well described by eq 1; fitting the frond data to the linear form of eq 1 gave \(r^2\) values \(\geq 0.95\). Doubling time, \(T_d\) (days), for duckweed growth in terms of frond numbers was calculated by use of

\[
T_d = \ln 2 / k
\]

equation (2)
The doubling times were lower (faster growth) for KCl-versus KCl with KCl or CaCl\(_2\), there were no significant differences in frond numbers among treatments. At day 7, in the KCl-amended systems, frond numbers with and without 2,4-DNT were not statistically different. In contrast, in treatments where 2,4-DNT was present, frond numbers in the CaCl\(_2\)-amended system were significantly lower than in the KCl + 2,4-DNT system. At subsequent times, differences between the CaCl\(_2\)+2,4-DNT and KCl + 2,4-DNT treatments remained significant with the later having higher frond numbers. By day 17, the difference in these two treatments was very large; 982 versus 76 fronds for the KCl + 2,4-DNT and CaCl\(_2\) + 2,4-DNT systems, respectively.

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The doubling times were lower (faster growth) for KCl treatments (\(T_d = 1.9\) days with 2,4-DNT and 2.0 days with no 2,4-DNT) relative to those for the CaCl\(_2\) treatments (5.0 days with 2,4-DNT and 2.7 days with no DNT), indicating the different degrees of bioavailability and toxicity of 2,4-DNT to duckweed over the course of incubation. In the key experiment, that is, the addition of CaCl\(_2\) to the potassium smectite system containing (mostly sorbed) 2,4-DNT, the effect of 2,4-DNT release (induced by Ca\(^{2+}\) exchange for K\(^{+}\)) on growth was evidenced by substantially decreased growth rate constant and increased doubling time compared with the effect of CaCl\(_2\) addition when no 2,4-DNT was present, and to all other treatments evaluated. The doubling time for the CaCl\(_2\)-amended potassium smectite system with 2,4-DNT present was 5.0 days compared to 1.9 days for the KCl-amended potassium smectite with 2,4-DNT present.

The results presented here clearly demonstrate that potassium smectite effectively sequesters 2,4-DNT, rendering it biologically unavailable in aqueous clay suspensions. Ion exchange of Ca\(^{2+}\) for K\(^{+}\) on potassium smectite-induced release of 2,4-DNT into solution where it severely inhibited growth of the aquatic plant duckweed. These findings could be useful in developing strategies for control of NAC mobility and bioavailability to target organisms such as plants and microorganisms utilized for in situ bioremediation technologies (39). As part of a watershed geostratification strategy, clay-modulated NAC sorption would allow sufficient phytoremediative plant biomass accumulation at otherwise phytotoxic contaminant levels. This itself would allow for effective phytostabilization and contaminated particulate erosion control. Furthermore, subsequent ion exchange of K\(^{+}\) (on clays) with Ca\(^{2+}\) could be used to release target NACs in a controllable fashion, that is, at nontoxic levels, into an active rhizosphere for effective plant uptake or bioremediation. Smectite clays are widely distributed in nature (1) and could be present at contaminated sites. This was indicated in a recent study where field-scale injections of K\(^{+}\) and Ca\(^{2+}\) electrolyte solutions were used to immobilize and remobilize NACs in a subsurface aquifer (40). Furthermore, smectites commonly occur as geologic deposits that are mined and used for many commercial, industrial, and environmental applications (41). Since they are readily available and inexpensive, smectite clays could realistically be added to soils, sediments, and surface waters to modulate the bioavailability and toxicity of NAC via the geochemical application described herein.

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**Literature Cited**


