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GEOCHEMICAL MODULATION OF BIOAVAILABILITY AND TOXICITY OF NITROAROMATIC COMPOUNDS TO AQUATIC PLANTS

By

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ABSTRACT

GEOCHEMICAL MODULATION OF BIOAVAILABILITY AND TOXICITY OF NITROAROMATIC COMPOUNDS TO AQUATIC PLANTS

By

MICHAEL GENE ROBERTS

Nitroaromatic compounds (NACs) are a prominent class of environmental toxic contaminants that pose a threat to human and ecosystem health. Nitroaromatic compounds demonstrate a high affinity with smectite clays which are widely distributed in nature as components of soils and sediments, and in geological deposits. The magnitude of NAC sorption by smectites is highly dependent on the exchangeable cation. For example, smectites saturated with K^+ have a very high affinity for NACs while those saturated with Ca^{2+} do not. In this study the ability of K^+-saturated smectite to attenuate the bioavailability and hence toxicity of 2,4-dinitrotoluene (2,4-DNT) to the aquatic plant duckweeds (*Lemna gibba* and *Lemna minor*) was evaluated. 2,4-DNT at the concentration of 7.5 mg/L was highly toxic to duckweeds. However, the presence of small amounts of K-smectite in aqueous growth media reduced this toxicity substantially because the sorption of 2,4-DNT by the added smectite reduced bioavailability. This reduced bioavailability could be modulated by simple K^+/Ca^{2+} cation exchange reaction occurring on mineral surfaces. In the subsequent experiments, $CaCl_2$ was added and the Ca^{2+} replaced K^+ associated with smectite surfaces. Such reaction released clay-sorbed NACs to aqueous solution, which is readily available to target plants. This study proves the concept that we can modulate the type and fraction of cations occupying the cation-

exchange-capacity sites of smectite clays to control the sorption/desorption hence bioavailability of NACs in smectite-rich soils and sediments. This information is needed in developing strategies for control of NAC mobility and bioavailability to target organisms such as plants and microorganisms utilized in *in-situ* bioremediation technologies.

DEDICATION

This work is dedicated to my daughter, Alyssa Marie, wife Jennifer, who have support and patienc has made this goal possible and my mother Annie Mae Roberts and family, who always sacrificed for my education. In the memories of my father, Clarence Roberts Sr. (July 16, 2005) and my grandmother, Susie Robinson Callier (January 23, 2006).

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CHAPTER I

Sorption of Nitroaromatics by Ammonium- and Organic Ammonium-Exchanged Smectite Shifts from Adsorption/Complexation to a Partition-Dominated Process

Abstract

Nitroaromatic compounds (NACs) are components of munitions commonly found as soil contaminants at military training sites and elsewhere. These compounds pose adverse effects on human health and ecological systems. Recent studies indicate these compounds are strongly retained by smectite clays. The adsorption mechanisms are not fully reconciled, but it is known that the type of exchangeable cation strongly affects NAC affinity for smectites. This study examined the sorption of 1,3-dinitrobenzene, 2,4-dinitrotoluene and naphthalene from water by a smectite clay (SWy-2) saturated with ammonium, tetramethylammonium (TMA), trimethylphenylammonium (TMPA) and hexadecyltrimethylammonium (HDTMA). In all cases, we observed greater sorption of 2,4-dinitrotoluene compared with 1,3-dinitrobenzene. The sorption isotherms for 2,4-dinitrotoluene and 1,3-dinitrobenzene displayed a concave-downward curve for NH_4-SWy-2 and TMA-SWy-2, whereas the isotherms for sorption of HDTMA-SWy-2 and TMPA-SWy-2 were essentially linear. The magnitude of sorption (sorbed 2, 4-DNT concentration at Ce = 40 mg/L in parenthesis) followed the order: NH_4-SWy-2 (40,165 mg/kg) > TMA-SWy-2 (23,370 mg/kg) > TMPA-SWy-2 (8,922 mg/kg) > HDTMA-SWy-2 (6,005 mg/kg) for both compounds. The greater affinity of NACs for NH_4 -and TMA-SWy-2 is due in part to complex formation between the exchangeable cation and –NO_2 groups. These clays also provide near optimal interlayer distances that approximate the molecular thickness of NACs hence promoting the simultaneous interaction of the planar aromatic rings with opposing siloxane surfaces, and solute dehydration. Both processes are energetically favorable. In HDTMA-SWy-2, sorption of all solutes is via a partition-dominated process. Solute competition (diminished uptake of the one solute in

the presence of a second one) was observed for TMA-SWy-2 but not HDTMA-SWy-2. This is consistent with an adsorptive mechanism for TMA-SWy-2 and a partitioning mechanism for HDTMA-SWy-2. This study demonstrates that the dominant molecular mechanism of NAC sorption by smectite changes fundamentally from complexation between $-NO_2$ groups and the exchangeable cation (viz. NH_4 and TMA) to partitioning for a systematic series of ammonium- and quaternary- ammonium cations in which the locus of positive charge (the central N atom) is progressively shielded by organic moieties of increasing size.

Introduction

Smectites are layered 2:1 aluminosilicates with structural negative charges that arise from isomorphous substitution. In nature, inorganic cations (e.g., Ca^{2+}, Mg^{2+}, Al^{3+}, K^+, and Na^+) are commonly associated with the clay surfaces to balance these negative charges. In the presence of water, inorganic exchangeable cations are hydrated causing the interlayer to expand. Among the clay minerals commonly found in soils, smectites are especially important because of their widespread occurrence, high surface areas, large cation exchange capacities, and high expansibility. Smectite clays can be modified through ion exchange reactions utilizing inorganic or organic cations. Previous studies have shown that these simple modifications can dramatically alter the affinities of smectites for organic contaminants and pesticides, and that this capability could enhance the successful implementation of a variety of in-situ and ex-situ remediation/immobilization technologies (Boyd et al., 1998a; 2001; Lee et al., 1998; Xu et al., 1997; Sheng et al., 1998; Weissmahr et al., 1999; Li et al., 2004a).

Replacing the inorganic exchangeable cations of smectites with organic cations typically causes an increase in the hydrophobic nature of the clay interlayer environment (Barrer, 1961; Boyd et al., 1988b; McBribe, 1977; Mortland et al., 1986; Wolfe, 1985; Xu and Boyd, 1995). For instance, as naturally occurring inorganic exchange cations of clay minerals are replaced with organic cationic surfactants such as hexadecyltrimethylammonium (HDTMA), the resultant organoclays are rendered more effective sorbents for removing poorly water soluble organic compounds from aqueous solution (Boyd et al., 1988; Burris, 1992; Lee et al., 1989; Sheng et al., 1996). In HDTMA-modified clays, the association of the C-16 alkyl carbon chains of HDTMA creates an organic phase in the clay interlayer that covers most clay siloxane surface. In the case of aromatic hydrocarbons, solute partitioning into this organic phase was shown to be the primary sorptive process (Jaynes and Boyd, 1991a). Increasing the amount of HDTMA in the clay interlayer enlarges the partitioning domains as indicated by an expansion of the clay basal spacing from 12 up to 18 and 23 Å. The increased interlayer distances of HDTMA-modified smectites correspond to the formation of bilayers and paraffin complexes by the alkyl-carbon chains of HDTMA.

Lee et al. (1989, 1990) showed that smectite clays exchanged with smaller organic cations such as tetramethylammonium (TMA) or trimethylphenylammonium (TMPA) cations display adsorptive properties as indicated by curvilinear isotherms for sorption of benzene and other aromatic hydrocarbons. They concluded that TMA and TMPA cations function as weakly hydrated pillars which prop open the smectite interlayers providing access to adsorption domains on the siloxane surface. Jaynes and Boyd (1991b) and Lee et al. (1990) studied the effect of clay surface charge density on adsorption of organic contaminants by such organo-smectites. High-charged smectites exchanged with a correspondingly greater amount of TMA or TMPA sorbed less benzene compared to TMA- or TMPA-modified smectites derived from clays with lower charge densities. The authors attributed this observation as additional evidence for organic solute adsorption by mineral siloxane surfaces between the exchangeable TMA or TMPA cations. Also, sorption of benzene vapor by dry TMA-smectite was greater compared to sorption of benzene from bulk water. Hydration of the TMA cations, albeit weak, diminished the size of adsorption domains between exchangeable TMA ions. Thus there are at least two primary mechanisms for sorption of organic solutes by organoclays, i.e., partitioning into organic phases derived from organic cations containing large alkyl hydrocarbon chains (e.g. HDTMA-clay), and adsorption to the available siloxane surfaces in organoclays comprised of smaller organic cations such as TMA.

So, for TMA-and TMPA-smectites the small, weakly-hydrated organic ammonium cations serve to prop open the clay interlayers without the organic cation or its hydration waters fully occupying the interlayer environment, hence rendering the clay siloxane surfaces available for adsorption of organic solutes. Recent studies have shown that several weakly-hydrated inorganic cations (e.g. K^+ and Cs^+) can manifest similar changes in the clay interlayer environment leading to higher adsorption of certain organic solutes (Haderlein and Schwarzenbach, 1993; Haderlein et al., 1996; Boyd et al., 2001; Sheng et al., 2002). For example, nitroaromatic compounds (NACs) demonstrate strong affinities for smectites exchanged with K^+ or Cs^+, but much less adsorption by smectites saturated with Na^+, Ca^{2+}, or Mg^{2+}. This was attributed to the comparatively stronger hydration of Na^+, Ca^{2+}, and Mg^{2+} cations which create an inhospitable (i.e. hydrophilic) environment for organics in clay interlayers. This is, in part, because strongly bound water is more difficult to displace, thus restricting access of the organic contaminants to potential adsorption sites in smectite.

The hydration of inorganic exchangeable cations strongly influences the interlayer environment in smectites where NAC sorption predominantly occurs. Besides determining the abundance of adsorptive sites of sufficient size (between exchangeable cations), interlayer cation hydration is a major determinant of the distance between clay layers in smectites. A d_{001} spacing of ~12.5 Å is commonly observed for K^+ and Cs^+-smectite clays. This appears to provide an optimal interlayer spacing which allows the planar aromatic rings of NACs to orient parallel to and interact directly with the opposing clay sheets, thereby, minimizing contact with water molecules, which is energetically favorable (Li et al., 2004b). This is consistent with the now common observation that smectite clays saturated with relatively weakly-hydrated K^+ or Cs^+ ions have a strong affinity for NACs (Boyd et al., 2001; Haderlein and Schwarzenbach, 1993; Haderlein et al., 1996; Johnston et al., 2001; Johnston et al., 2002; Li et al., 2004b; Sheng et al., 2002; Weissmahr et al., 1998). In contrast, those saturated with more strongly hydrated cations, such as Na^+ or Ca^{2+}, are much less effective adsorbents for NACs. Such an arrangement of NACs in the interlayers of K^+-, Cs^+- or NH_4^+-smectites might also allow the formation of electron donor-acceptor complexes involving the electron deficient aromatic ring of NACs and the electron rich siloxane oxygens of smectites (Haderlein and Schwarzenbach, 1993; Haderlein et al., 1996; Weissmahr et al., 1998). Weakly-hydrated exchangeable cations on clay surfaces can also favorably interact with the $-NO_2$ groups of NACs. Boyd et al. (2001) demonstrated that one mechanism responsible for the strong sorption of NACs is the formation of inner sphere and/or outer sphere complexes with weakly-hydrated exchangeable cations in the clay interlayers.

Smectite clays are strong sorbents for many organic compounds. The dominant sorption mechanism (s) on a given smectite varies depending on properties of the exchangeable cations and the organic solutes. Previous studies indicated that organic compounds could be retained by partition interactions with organic phases derived from exchanged organic cations and by adsorption on clay siloxane surfaces. The latter process results from additive processes including hydrophobic interactions between the solute and the clay siloxane surfaces, solute dehydration in the clay interlayers, formation of complexes between polar functional groups of the solute and exchangeable cations and/or formation of electron-donor acceptor complexes. The size, structure, and hydration properties of exchangeable cations associated with smectites strongly influence the clay interlayer environment, hence determining the degree of sorption of organic compounds and the operative sorption mechanism(s). To further understand the dominant sorptive mechanism(s) of smectite clays responsible for the retention of NACs where multiple mechanisms are possible, we measured the sorption from water of 1,3-dinitrobenzene (1,3-DNB) and 2,4-dinitrotoluene (2,4-DNT) and naphthalene in single- and binary-solute systems by a reference smectite (SWy-2) saturated with ammonium and a series of organic quaternary ammoniums, viz. TMA, TMPA and HDTMA, of increasing size and hydrophobicity. A central goal of this study was to determine the predominance of complex formation between the exchangeable cation and the $-NO_2$ groups of NACs (Boyd et al., 2001) as the locus of positive charge, viz. the central N atom, is progressively shielded by organic moieties of increasing size (methyl, phenyl, hexadecyl). XRD and surface area determination of clays were used to gain insight into the effects of exchangeable cation on the nature of the clay interlayer environment and hence sorption of NACs.

Materials and Methods

Chemicals

1,3-DNB and 2,4-DNT were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI) with a reported purity > 97%, and naphthalene was obtained from Sigma Chemical Company Inc. (St. Louis, MO) with a reported purity of 99%. Methanol (HPLC grade) was purchased from Millinckrodt Baker Inc. (Phillipsburg, NJ). The HDTMA-bromide, TMA-bromide, TMPA-chloride, and NH_4-chloride were purchased from Aldrich with chemical purities > 98%. All chemicals were used as received.

Clay Preparation

A reference smectite clay (Wyoming montmorillonite SWy-2) was obtained from the Source Clays Repository of Clay Minerals Society at Purdue University (West Lafayette, IN) and used throughout this study. The SWy-2 has a cation exchange capacity (CEC) of 82 $cmol_c/kg$ and a theoretical surface area of 750 m^2/g (van Olphen and Fripiat, 1979). The < 2-μm particle-sized clay fraction was obtained by wet sedimentation. The clay suspensions were then quick frozen and freeze-dried. Cation exchange reactions were used to prepare the homoionic clays. The naturally occurring inorganic exchangeable cations were replaced by ammonium, TMA, TMPA and HDTMA. The NH_4-smectite was prepared by washing the clay three times with 0.2 M of NH_4Cl solution. The TMA- and TMPA clays were prepared by adding TMPA chloride and TMA bromide in excess of three times the clay CEC. HDTMA-saturated clays were prepared by adding HDTMA bromide equal to the clay CEC. Then the clay suspensions were washed four to five times with Milli-Q water to remove excess electrolytes; $AgNO_3$ was used to indicate if the samples were free of chloride or bromide. The clay suspensions were then quick-frozen and freeze-dried. The clay organic carbon content was measured using combustion method and surface area was determined using N_2-BET method.

Single-Solute Sorption

The sorption of 1,3-DNB, 2,4-DNT and naphthalene by NH_4-, TMA-, TMPA- and HDTMA-SWy-2 was measured using a batch equilibration method. First 0.05 to 0.10 g clay samples depending on the type of solute and prepared clay were placed in glass centrifuge tubes. Then added a series of initial solute concentrations in water ranging from 5 to 200 mg/L for 1,3-DNB and 2,4-DNT and 1.0 to 25 mg/L for naphthalene. The tubes were closed immediately with Teflon-lined screw caps and shaken end-over-end at 40 rpm for 24 h at room temperature (23 °C). Previous studies demonstrated that sorptive equilibrium in such systems has been reached within 18 h (Sheng et al., 2002). The tubes were centrifuged at 2600 g for 30 min to separate liquid and solid phases. Concentrations of 1,3-DNB and 2,4-DNT and naphthalene in supernatants were measured by a high-performance liquid chromatography (HPLC) system consisting of a Perkin-Elmer Binary 250 LC pump, a Series 200 autosampler and a Series 200 UV-visible detector. The optimal wavelength was set at 270 nm for 1,3-DNB, 250 nm for 2,4-DNT and 260 nm for NAPH. The mobile phase composition was a mixture of methanol and water, and optimized for each compound. Controls consisted of the aqueous organic solutes in the absence of clay. The sorbed amount was calculated from the concentration difference between the aqueous solute concentrations in the control and supernatants in the clay suspensions. Sorption isotherms were obtained by plotting sorbed vs. equilibrium aqueous phase concentrations. No changes in solute concentrations were detected in the control tubes, therefore solute mass lost from the aqueous phase in the presence of clay was assumed to be sorbed by clay.

Binary-Solute Sorption

To evaluate the competitive sorption of NACs by clays, we conducted binary-solute sorption experiments using the batch equilibration method. A series of initial solutions were prepared by mixing 2,4-DNT and 1,3-DNB with the molar ratios of 1:1 and 1:3 in aqueous solution. These initial solutions were added to centrifuge tubes containing HDTMA-SWy-2 or TMA-SWy-2. The equilibration and clay-solution separation procedures were the same as described above. The supernatants were sampled and subjected to simultaneous measurements of 1,3-DNB and 2,4-DNT by HPLC. The mobile phase was an isocratic mixture of methanol and water (50:50) with the flow rate at 1.0 ml/min. The wavelength of UV-vis detector was set at 270 nm.

X-ray Diffraction Analysis

After the supernatants were removed for HPLC analysis, approximately 2 mL of clay suspension remained in the tubes. The clay slurries were mixed using a vortex mixer, dropped onto glass slides using a disposable glass pipette, air-dried to produce oriented clay films, then subjected to X-ray diffraction (XRD) analysis. XRD patterns of clay films were recorded using a Philips APD 3720 automated X-ray diffraction meter equipped with Cu-Kα radiation, an APD 3521 goniometer and a diffracted-beam monochromator. The scanning angle (2θ) ranged from 3 to 15° at steps of 0.02°, and the scanning time was 2 seconds per step.

Results and Discussion

The properties of the clays used in this study are presented in Table 1. Reference SWy-2 and NH_4-SWy-2 clays each contained ~ 0.1 % organic carbon, presumably present as an impurity. The organic carbon values of the organoclays increased in the expected order: TMA-SWy-2 < TMPA-SWy-2 < HDTMA-SWy-2. Comparisons of measured organic carbon content with the calculated organic carbon percentages at full saturation indicated that 76.5, 83 and 83% of the cation exchange sites were occupied by organic ammonium cations for TMA-SWy-2, TMPA-SWy-2 and HDTMA-SWy-2, respectively. The basal spacing (d001) increased with the size of the predominant exchangeable cation: NH_4-SWy-2 < TMA-SWy-2 < TMPA-SWy-2 < HDTMA-SWy-2. For the organoclays, the measured N_2 BET surface areas were inversely proportional to the size of the organic ammonium, i.e., HDTMA-SWy-2 < TMPA-SWy-2 < TMA-SWy-2. This ordering results from the fact that the larger organic ammonium cations occupy a greater portion of the interlayer volume and obscure a larger portion of the siloxane surfaces where N_2 adsorption occurs. This also accounts for why the measured N_2 BET surface areas of the organo-clays are less than the theoretical surface area of SWy-2.

Table 1. Properties of reference smectite (SWy-2) and SWy-2 exchanged with ammonium, trimethylammonium (TMA), trimethylphenylammonium (TMPA) and hexadecyltrimethylammonium (HDTMA).

Clay	% Organic Carbon	% CEC Occupied by Organic Cation*	N_2 BET Surface Area (m^2/g)	d_{001} (Å)
Reference smectite (SWy-2)	0.1	___	21.3	12.0
NH_4-SWy-2	0.1	___	43.4	11.2
TMA-SWy-2	3.0	76.5	132.3	13.5
TMPA-SWy-2	7.4	83	19.5	14.2
HDTMA-SWy-2	15.7	83	13.7	17.5

Percentage of CEC occupied by organic carbon was calculated by the measured organic carbon content divided by the organic carbon from organic cations assuming CEC sites are fully saturated with organic cations.

Sorption of Nitroaromatic Compounds

Sorption isotherms of 2,4-DNT and 1,3-DNB by HDTMA-, TMPA-, TMA- and NH_4-SWy-2 from water are shown in Figure 1 with amount sorbed per unit mass of clay plotted vs. the equilibrium aqueous concentration of the solute. All sorption isotherms were fit to the Freundlich equation (shown as solid lines in Fig.1):

$$Q = K_f Ce^N$$

where Q (mg/kg) is the sorbed concentration, Ce (mg/L) is the equilibrium aqueous concentration, K_f ($mg^{1-N}L^N/kg$) is the Freundlich sorption coefficient and N is an empirical constant (unitless) that describes isotherm nonlinearity. The Freundlich equation fitting parameters are listed in Table 2.

Sorption isotherms of 2,4-DNT and 1,3-DNB by the clays were nonlinear except that representing sorption of 2,4-DNT by HDTMA-SWy-2. The magnitude of sorption on clays was greater for 2,4-DNT than for 1,3-DNB. The higher affinity of clays for 2,4-DNT compared to 1,3-DNB is likely due in part to the lower water solubility of the toluene derivative. Our previous studies of NAC sorption by K-SWy-2, and aromatic hydrocarbon sorption by HDTMA-smectites, have shown that solute uptake from water is favored by lower water solubility (Boyd et al., 2001; Li et al., 2004, Jaynes and Boyd, 1991a). The sorption of 1,3-DNB and 2,4-DNT by clays followed the order of NH_4-SWy-2 > TMA-SWy-2 > TMPA-SWy-2 > HDTMA-SWy-2. The strong affinities of the NACs for NH_4-SWy-2 and TMA-SWy-2, along with the sorption nonlinearity, are characteristics of adsorption on the basal surfaces of smectite clays. Compared to naturally occurring inorganic exchangeable cations commonly associated with smectites (e.g. Na^+, Ca^{2+}, Mg^{2+}), the relatively low hydration free energies for ammonium (-292 kJ/mol) (Marcus, 1985) and TMA (-219kJ/mol)(Ford and Wang, 1992) result in less water surrounding the cations rendering more mineral siloxane surface available for adsorption (Boyd et al., 2001). The NH_4-SWy-2 was at least 1.5 times more effective (on a unit mass basis) than TMA-SWy-2 for sorption of 2,4-DNT and 1,3-DNB over the concentration range studied. The higher affinity of NH_4-SWy-2 for these solutes may result from the formation of complexes between exchanged ammonium and the $-NO_2$ groups of the NACs, as in the case of NAC sorption by K-smectites (Boyd et al., 2001). The larger molecular size of TMA than that of ammonium would inhibit direct interactions between $-NO_2$ and TMA ions since the locus of positive charge in TMA is buried inside four methyl groups. Thus $-NO_2$ complexes with ammonium would be expected to be stronger than those with TMA, leading to greater NAC sorption by NH_4-SWy-2.

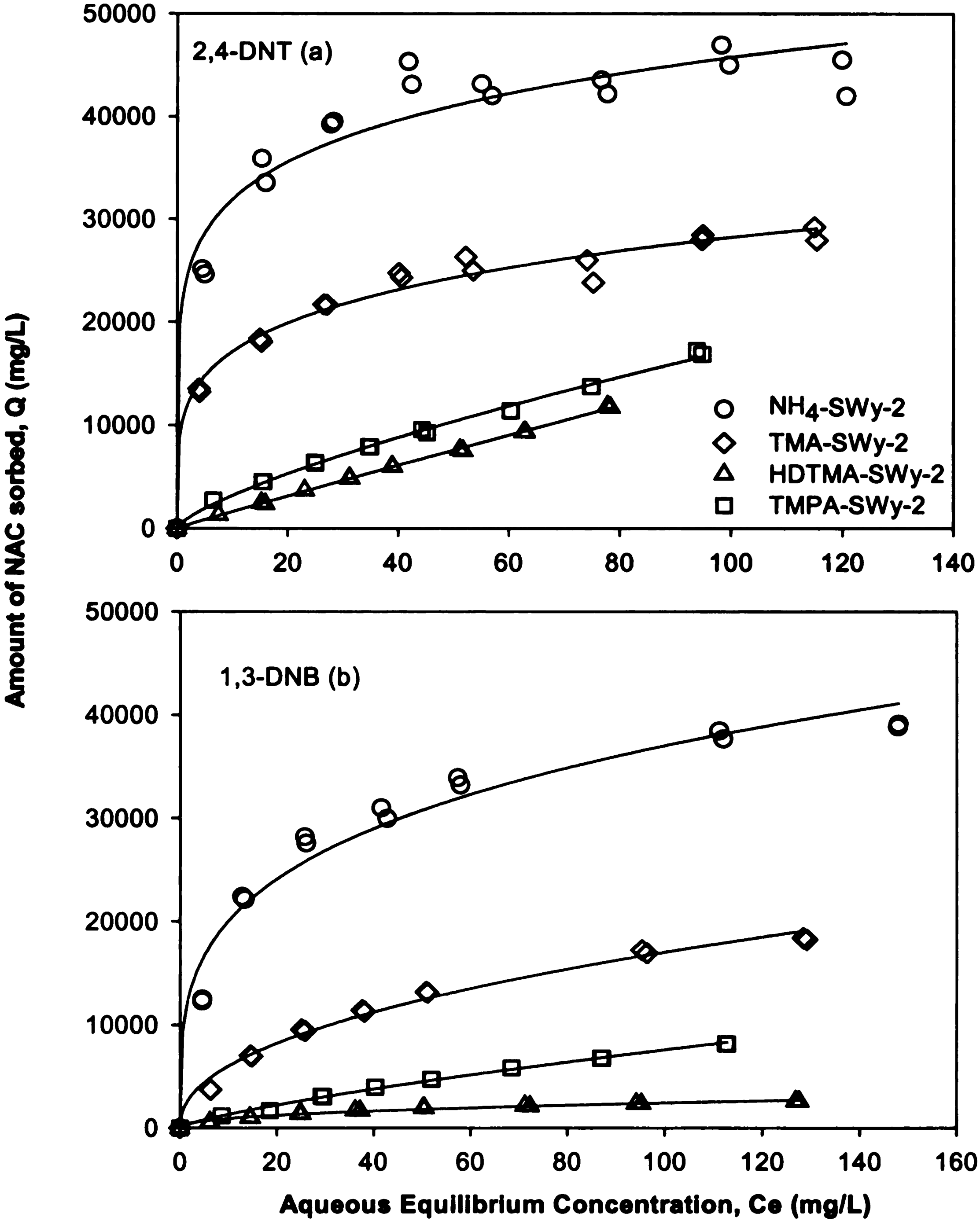

Figure. 1. Sorption of 2,4-dinitrotoluene (2,4-DNT) and 1,3-dinitrobenzene (1,3-DNB) by smectites saturated with ammonium (NH_4), tetramethylammonium(TMA), trimethylphenylammonium (TMPA) or hexadecyltrimethylammonium (HDTMA).

2,4-DNT and 1,3-DNB molecules differ by only a methyl group. The weaker sorption by NH_4- and TMA-SWy-2 observed for the smaller molecule 1,3-DNB than 2,4-DNT cannot be explained by steric hindrance or surface-filling arguments. Rather, it likely results from stronger complexes between 2,4-DNT forms and surfaces and/or cations. The methyl group is expected to donate electron density to the ring, which should decrease the strength of electron-donor-acceptor complexes with the surface as proposed by Haderlein et al. (1996). In fact, much of the donated electron density would probably flow to the nitro groups in para- and ortho-positions (Boyd et al., 2001), which may enable stronger formation of complexes with interlayer cations. Another factor that helps to account for the stronger sorption of 2,4-DNT over 1,3-DNB is that hydrophobic effects make a contribution to the sorption of NACs (Boyd et al., 2001; Li et al., 2004b). 2,4-DNT is less water-soluble than 1,3-DNB so that there should be more impetus to remove it from water.

Sorption of NACs from water by HDTMA-and TMPA-clays exhibited sorption properties distinct from those of NH_4- and TMA-clays. Sorption of NACs by HDTMA- or TMPA-clays was lower than sorption by NH_4- or TMA-SWy-2 clays and the isotherms were more linear (Table2 and Fig 1). In HDTMA-SWy-2, the association of alkyl chains creates an organic partitioning phase that covers most clay siloxane surfaces; solute partitioning into this organic phase is believed to be the primary process occurring in this clay for aromatic hydrocarbons (Boyd et al., 1988; Jaynes and Boyd 1991a). The linear isotherms and inverse relationship between sorption and solute water solubility are consistent with a partition-dominated process (Chiou, 2002). The basal spacing of HDTMA-SWy-2 used herein was 17.5 Å, corresponding to the formation of a bilayer of alkyl chains (Jaynes and Boyd, 1991a). In this configuration, the densely packed alkyl chains adopt a horizontal orientation relative to clay layers forming an organic partition phase in which mineral surfaces are largely covered and hence contribute minimally to the sorption of organic compounds. Sorption of NACs by TMPA-smectite is slightly higher than that by HDTMA-clay, which may be due to some contribution of interlayer surfaces to adsorption. Overall, sorption by the TMPA-clay is intermediate between HDTMA-clay where NAC sorption is predominantly via partitioning and TMA-clay which functions as an adsorbent. The underlying mechanism(s) for NAC sorption by TMPA-clay is uncertain.

Table 2. Freundlich sorption isotherm parameters K_f (mg $^{1\text{-}N}$ L^N/ kg), N and r^2 for 2,4-dinitrotoluene (2,4-DNT), 1,3-dinitrobenzene (1,3-DNB) and naphthalene (Naph) by smectite clay (SWy-2) exchanged with ammonium, tetramethylammonium (TMA), trimethylphenylammonium (TMPA) and hexadecyltrimethylammonium (HDTMA). Standard errors are reported in parentheses.

	NH_4-SWy-2			TMA-SWy-2			TMPA-SWy-2			HDTMA-SWy-2		
Solute	K_f	N	r^2	K_f	N	r^2	K_f	N	r^2	K_f	N	r^2
1,3-DNB	10860 (940)	0.27 (0.02)	0.97	2120 (180)	0.45 (0.02)	0.99	228 (21)	0.27 (0.02)	0.97	331 (29)	0.43 (0.02)	0.98
2,4-DNT	22260 (1760)	0.16 (0.02)	0.95	10380 (610)	0.22 (0.01)	0.98	582 (44)	0.74 (0.02)	0.99	174 (9)	0.96 (0.02)	0.99
Naph	3.1 (1.2)	1.30 (0.17)	0.97	11.7 (5.74)	3.28 (0.37)	0.95	23527 (747)	1.14 (0.07)	0.98	331 (41)	1.39 (0.03)	0.99

Naphthalene Sorption

In contrast to the sorption order of NACs, the magnitude of naphthalene sorption followed the order: TMPA-SWy-2 > HDTMA-SWy-2 > TMA-SWy-2 > NH_4-SWy-2 (Figure 2). As we observed previously (Jaynes and Boyd, 1990) TMPA- SWy-2 appeared to have an unusually high affinity for naphthalene. In all cases, sorption of naphthalene by clays produced upward curvature in the isotherms (Figure 2 and Table 2) indicating that the increased concentration of naphthalene promotes further sorption in the interlayers. Hundal et al. (2001) have suggested that sorption of phenanthrene by smectites was primarily via a process of capillary condensation in interlayer nanopores and micropores; phenanthrene was physically entrapped in the nanopores and micropores of the clay driven primarily by hydrophobic effects. Zhu et al. (2004) have shown a strong correlation between Freundlich sorption constants and indices of cation-π bonding. They suggested that cation-π bonding occurred between polycyclic aromatic hydrocarbons and inorganic exchangeable cations in smectite interlayers. A comparison of sorption between the two types of compounds in this study (naphthalene and NACs) by organoclays suggests the prepared organoclays possess different sorption sites/mechanisms for retention of the test compounds. The much stronger affinity of NH_4-, and TMA-SWy-2 for NACs compared to naphthalene indicates that interlayer adsorption on the siloxane surfaces and complex formation involving the $-NO_2$ groups are the predominant sorptive interactions for polar NACs; the latter mechanism is not available for adsorption of nonpolar naphthalene. In contrast, TMPA-SWy-2 has a particularly high affinity for naphthalene with upward curvature of the isotherm, suggesting capillary condensation as a possible mechanism. Regardless of sorption mechanisms, it seems plausible that the aromatic groups on TMPA would prop the smectite interlayer at a d-spacing (14.2 Å, Table2) that might be optimal for naphthalene.

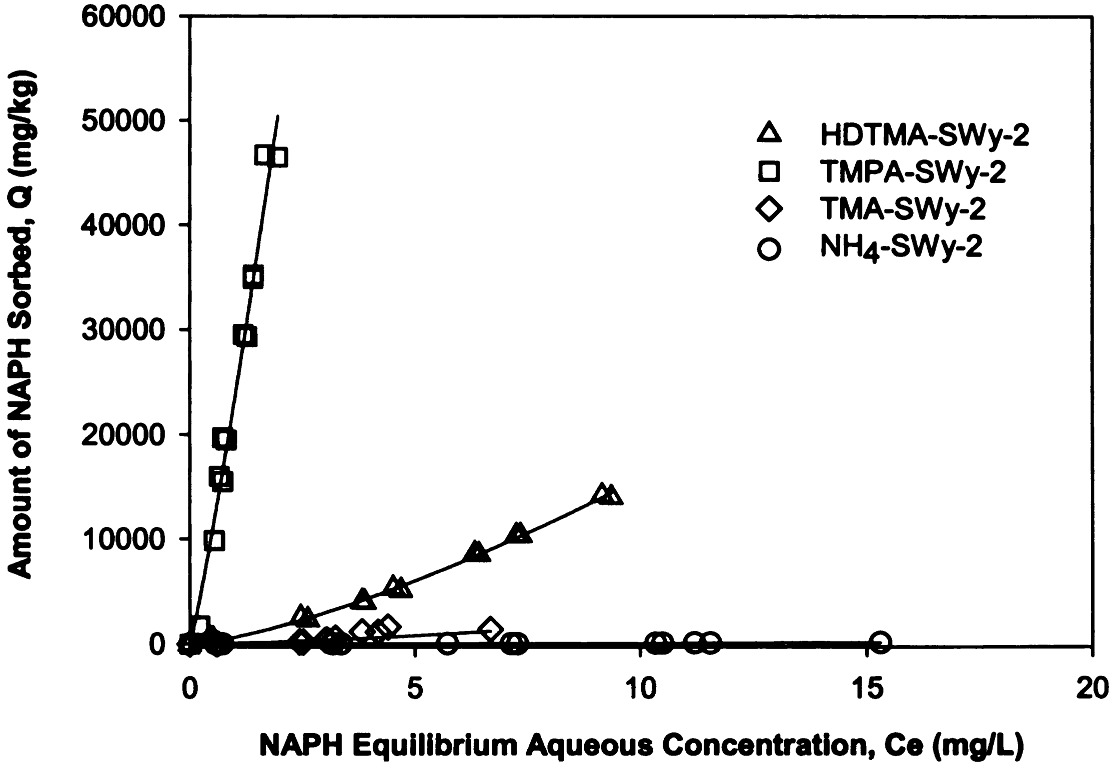

Figure 2. Sorption isotherms of naphthalene (NAPH) by smectite (SWy-2) saturated with ammonium, tetramethylammonium (TMA), trimethylphenylammonium (TMPA) or hexadecyltrimethylammonium (HDTMA)

Sorption from binary solute-mixtures

Sorption of NACs from binary solute mixtures by clays was measured to evaluate solute competition as a means of further revealing the operative sorption mechanisms. Isotherms representing sorption of a binary mixture of 1,3-DNB and 2,4-DNT from water by HDTMA-SWy-2 and TMA-SWy-2 are shown in Figures 3 and 4 along with the corresponding single solute sorption isotherms. The Freundlich fitting parameters are reported in Table 3. The binary solutions of 2,4-DNT and 1,3-DNB were prepared at initial molar ratios of 1:1 and 1:3. Sorption isotherms of 2,4-DNT from the binary solute mixtures by HDTMA-SWy-2 were coincident with the single solute isotherm demonstrating a total lack of solute competition. Sorption of 1,3-DNB in binary solute mixtures by HDTMA-SWy-2 manifested somewhat higher sorption compared to the single solute sorption at Ce > 50 mg/L, indicating a cooperative sorption process. The lack of a negative effect on sorption in binary solute systems is consistent with a partition-dominated process involving the organic phase formed by HDTMA (Chiou, 2002)

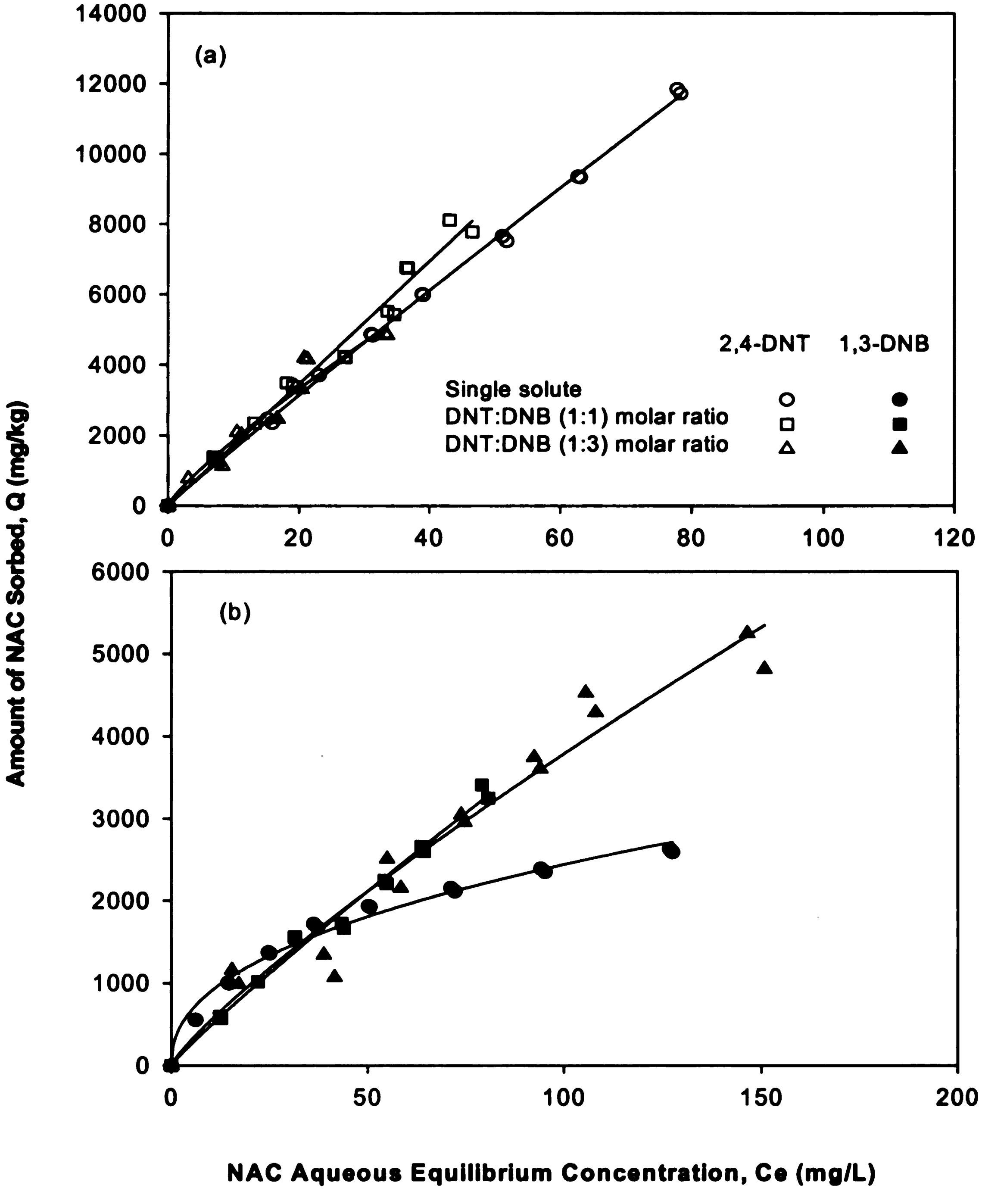

Figure 3. Sorption isotherms of nitroaromatic compounds (NACs) (a) 2,4-dinitrotoluene (2,4-DNT) and (b) 1,3-dinitrobenzene (1,3-DNB) by hexadecyltrimethylammonium (HDTMA)-smectite (SWy-2) in single- and binary-solute systems.

Sorption of 1,3-DNB and 2,4-DNT from binary solute mixtures by TMA-SWy-2 are compared to the sorption of the single solutes in Figure 4. Sorption isotherms of 1,3-DNB and 2,4-DNT in both single and binary solute systems are nonlinear indicating adsorption to clay surfaces rather than a partitioning process, as described above. In both cases, the presence of a second solute depressed the uptake of the primary solute clearly demonstrating that 1, 3-DNB and 2,4-DNT were competing for adsorptive sites on TMA-clay. The strong competitive effect on sorption of NACs by TMA-clay is characteristic of an adsorptive process wherein 1,3-DNB and 2,4-DNT compete with each other for the limited available sorptive sites. The greater amount of competitive solute present in aqueous phase was observed to more substantially suppress the sorption of the primary solute. For example, at Ce = 80 mg/L, binary sorption of 1,3-DNB was reduced ~ 50% with the DNT: DNB molar ratio of 1:3, and ~70% at a molar ratio of 1:1. In comparing the affinity of 1,3-DNB and 2,4-DNT with TMA-SWy-2, note that 2,4-DNT suppressed sorption of 1,3-DNB by up to 75%, while 1,3-DNB suppressed 2,4-DNT sorption by less than 30%. The greater competitive inhibition on sorption caused by 2,4-DNT is a logical manifestation of its higher affinity for TMA-SWy-2 compared to 1,3-DNB (Fig 1).

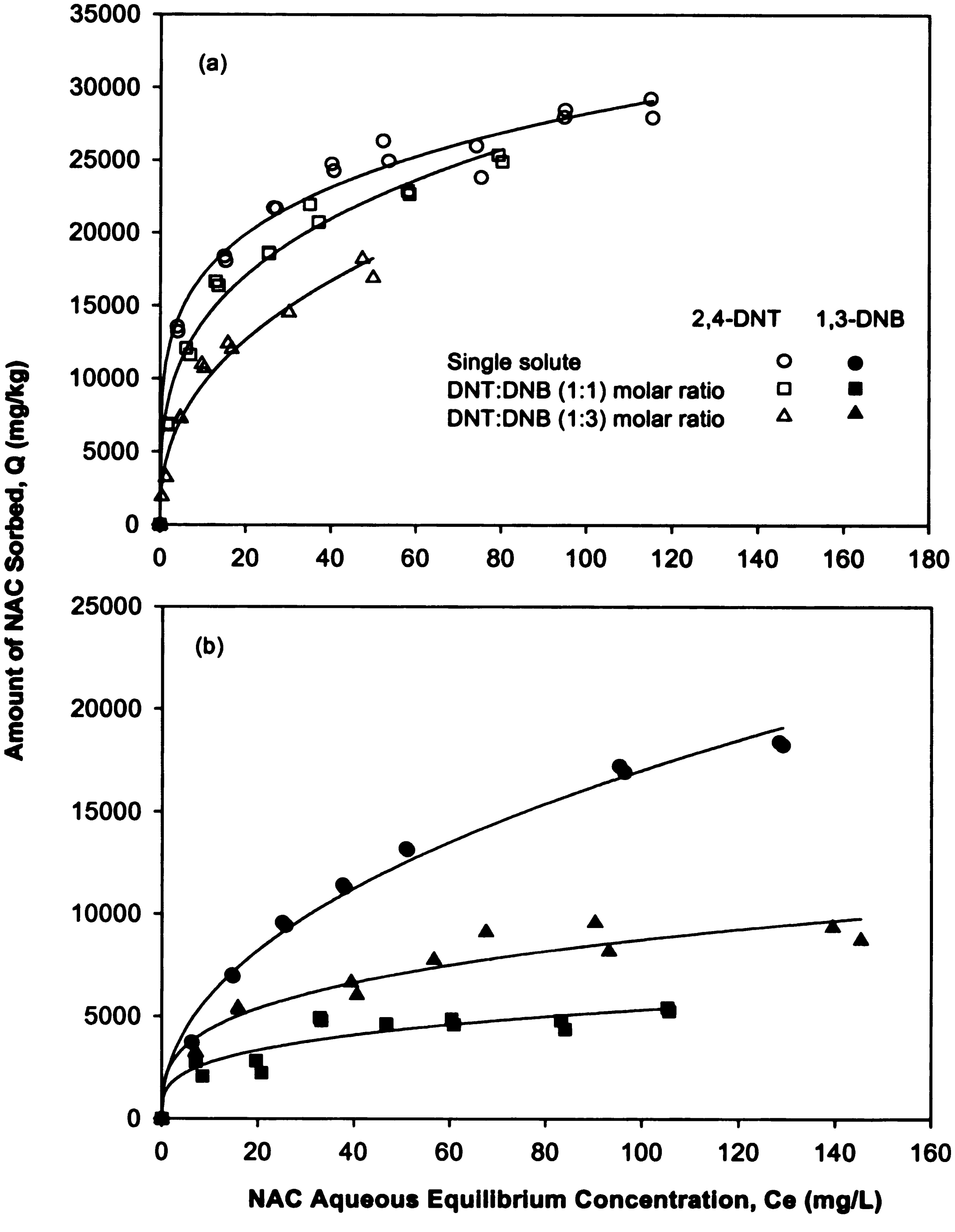

Figure 4. Sorption isotherms of nitroaromatic compounds (NACs) (a) 2,4-dinitrotoluene (2,4-DNT) and (b) 1,3-dinitrobenzene (1,3-DNB) by tetramethylammonium (TMA)-smectite (SWy-2) in single- and binary-solute syste

Table 3. Freundlich sorption isotherm parameters K_f (mg $^{1-N}$ L^N/ kg), N and r^2 for 1,3-DNB and 2,4-DNT by tetramethylammonium (TMA)- and hexadecyltrimethylammonium (HDTMA)-smectite (SWy-2) in binary solute systems. The standard errors are reported in parentheses.

Solute System	TMA-SWy-2			HDTMA-SWy-2		
	K_f	N	r^2	K_f	N	r^2
1,3-Dinitrobenzene (1,3-DNB)						
Molar Ratio DNT: DNB (1:1)	1867 (216)	0.23 (0.03)	0.96	58 (10)	0.92 (0.04)	0.99
DNT: DNB (1:3)	2950 (714)	0.22 (0.06)	0.85	79 (26)	0.84 (0.07)	0.96
2,4-Dinitrotoluene (2,4-DNT)						
Molar Ratio DNT: DNB (1:1)	6252 (353)	0.33 (0.01)	0.99	168 (36)	1.0 (0.06)	0.98
DNT: DNB (1:3)	3790 (310)	0.40 (0.03)	0.98	270 (63)	0.84 (0.07)	0.96

Summary

Sorption of two NACs by smectite exchanged with ammonium and a series of quaternary ammonium ions (TMA, TMPA, HDTMA) revealed a shift in sorption mechanism from a predominately adsorptive process in the case of NH_4- and TMA-smectite to a partition-dominated process for HDTMA-smectite. Nonlinear sorption isotherms and competition between NACs for sorption sites indicated an adsorptive mechanism. Adsorption of NACs was a manifestation of the small size and low hydration energies of ammonium and TMA. The primary adsorptive interaction appeared to be the formation of complexes between exchangeable cation and the $-NO_2$ groups of the NACs. Adsorption domains are primarily the siloxane surface between the exchangeable cations. The small size and weak hydration of these cations (NH_4^+, TMA) facilitates formation of the aforementioned complexes and makes available the siloxane surface, which is largely unobscured by waters of hydration associated with the exchangeable cations. Solute partitioning was the primary sorption mechanism of clay exchanged with the much larger and hydrophobic cation HDTMA. In this instance, the C-16 alkyl chains of exchanged HDTMA coalesce to form a hydrophobic partition phase into which NACs dissolve. The siloxane surfaces and interlayer volume is essentially fully occupied by exchanged HDTMA. Linear isotherms and lack of solute competition in binary-solute systems provide evidence for a partition-dominated process where degree of uptake from water is determined mostly by the water insolubility of the solute.

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CHAPTER II

REDUCING BIOAVAILABILITY AND PHYTOTOXICITY OF NITROAROMATIC COMPOUNDS BY SORPTION ON K-SMECTITE CLAY

Abstract

Nitroaromatic compounds (NACs) are a prominent class of environmental contaminants that are toxic to plants and microorganisms, and pose a threat to human and ecosystem health. The NACs demonstrate high affinities for smectite clays which are widely distributed in nature as components of soils and sediments, and in geological deposits. The affinity of NACs for smectites is highly dependent on the exchangeable cation. For example, smectites saturated with K^+ have very high affinities for NACs while those saturated with Ca^{2+} do not. In this study we evaluate the ability of K-smectites to attenuate the bioavailability and hence toxicity of 2,4-dinitrotoluene (2,4-DNT) to the aquatic plant duckweed (*Lemna gibba* and *Lemna minor*). In the absence of K-smectite, 7.5 mg/L of 2,4-DNT was highly toxic to duckweed. Small amounts of K-smectite added to aqueous growth media reduced this toxicity substantially, presumably by reducing 2,4-DNT bioavailability via sorption. There is a need to optimize bioavailability of NACs during bioremediation so that they are sufficient available for effective plant uptake or microbial degradation without being present at levels that are toxic to organisms used for bioremediation. Release of clay-bound NACs can be achieved by simple ion exchange reactions on the clays.

Introduction

Smectites are layered 2:1 aluminosilicates with structural negative charges arising from isomorphous substitution in octahedral and/or tetrahedral sheets. In nature, inorganic cations (e.g., Ca^{2+}, Mg^{2+}, Al^{3+}, K^+, and Na^+) are associated with smectite clay surfaces to balance these negative charges. When smectites are wetted, these cations hydrate causing greater separation of the clay sheets (interlayer expansion). Among the clay minerals commonly found in soils, smectites are especially important as potential adsorbents of organic contaminants and pesticides because of their widespread occurrence (Allen and Hajek, 1989), high surface areas, large cation exchange capacities, and reversible interlayer expansibility.

Sorption to soils and sediments is a major determinant of environmental fate and impact of contaminants, such as potential for leaching and bioavailability to microorganisms and plants (Koskinen and Harper, 1990). Sorption often renders contaminants less available to receptor organisms (Hatzinger and Alexander, 1995; Luthy et al., 1997; Ogram et al., 1985; Pignatello and Xing, 1996). Aqueous contaminant concentrations are lowered by sorption, thereby inhibiting root uptake by plants. Similarly, soil microorganisms may ineffectually degrade organic contaminants if they cannot readily access the sorbed contaminant pool. Harris (1964) showed that insecticide adsorption by soil minerals under dry conditions substantially raised the lethal dose (LD_{50}) towards crickets, whereas hydration caused release of sorbed contaminants hence increasing bioavailability of the insecticides. As a consequence, lowered LD_{50} values were observed for insecticides added to wet soils compared to the same soils in a dry state. Steinberg et al. (1987) and Scribner et al.

(1992) showed that sorption and sequestration of ethylene dibromide and simazine pesticides in field-weathered soils reduced bioavailability to contaminant-biodegrading microorganisms thereby prolonging pesticide residence times. Reduced bioavailability in these instances was associated with prolonged pesticide-soil contact times. In contrast, there are examples where bacteria are able to access directly the pool of sorbed substrate (Feng et al., 2000; Guerin and Boyd, 1992; Park et al., 2004). So, it is unclear, *a priori*, whether or not sorbed contaminants are available to biological organisms. At this point, no generalizations regarding the bioavailability of sorbed organic contaminants are apropos.

Nitroaromatic compounds (NACs) are a class of prominent soil contaminants that are toxic to plants and microorganisms (Gong et al., 1999; Sunahara et al., 1998). Nitroaromatic compounds demonstrate high affinity for certain clays, especially smectites, which is strongly influenced by the type of cation occupying cation exchange sites of the clay. For example, smectites exchanged with Cs^+, K^+ and NH_4^+ have high affinities for NACs whereas smectites manifest much lower NAC adsorption when exchanged with Na^+, Ca^{2+} and Mg^{2+} (Boyd et al., 2001; Haderlein and Schwarzenbach, 1993; Haderlein et al., 1996; Johnston et al., 2001; Johnston et al., 2002; Li et al., 2004; Sheng et al., 2002). The effect of exchangeable cation type on NAC sorption appears to be related to their hydration energies. The presence of strongly hydrated exchangeable cations creates an unfavorable clay interlayer environment for NAC adsorption, whereas the comparatively weak hydration of other exchangeable cations creates a much more favorable interlayer environment for NAC adsorption. Adsorption of NACs occurs primarily in the clay interlayer (Boyd et al., 2001; Sheng et al., 2002), and cation hydration determines the interlayer distance, size of adsorptive domains, and ability to form NAC-cation complexes.

Boyd and co-workers (Boyd et al., 2001; Johnston et al., 2001; Li et al., 2004a; Sheng et al., 2002) conducted systematic studies on the adsorption mechanisms of NACs by smectite clays using a series of complimentary investigatory techniques including detailed macroscopic sorption measurements, Fourier transform infrared spectroscopy, X-ray diffraction, quantum calculations, molecular dynamics simulations, and thermodynamic measurements. Their results revealed that weaker hydration of exchangeable cations (e.g., K^+) on smectite surfaces promotes favorable interactions between cations and the $-NO_2$ groups of NACs via formation of inner- and/or outer-sphere complexes. In addition to the formation of such complexes, an optimal basal distance of ~ 12.5 Å was observed for K^+-smectite clays (Li et al., 2004a; Sheng et al., 2002). This allowed simultaneous interactions of the planar aromatic rings of intercalated NACs with the opposing clay siloxane surfaces, as well as partial solute dehydration; both processes are energetically favorable (Li et al., 2004a). Furthermore, compared with cations having higher hydration enthalpies, e.g. Ca^{2+}, Mg^{2+} or Na^+, the smaller hydration spheres surrounding Cs^+, K^+ or NH_4^+ provide larger regions of siloxane surface between exchange cations that are unobscured by water. These siloxane surface regions are the primary adsorptive domains where NACs align in an orientation parallel to the clay sheets.

Nitroaromatic compounds are common components of munitions and are commonly found as soil and ground water contaminants in military training facilities and manufacturing sites. Important classes of pesticides such as the dinitrophenol herbicides may persist in soils where they are used in crop protection (Broholm, 2001; Hawley, 1981), leaching to contamination of surface and ground waters. For these reasons, NACs pose the threat of adverse effects on human and ecosystem health (Berthe-Corti et al., 1998; Gong et al., 1999; Hawley, 1981; Neumann et al., 1995). The $-NO_2$ groups of absorbed NACs can also be reduced to amino groups that can conjugate with the phenolic sites in biota (Morgan, 1989; Ware, 1997).

Understanding the sorption mechanism(s), and the molecular scale forces and properties of smectite clays that influence sorption, provides the scientific basis for developing protocols to control the mobility and bioavailability of NACs to target organisms such as plants and microorganisms utilized in bioremediation technologies. Li et al., (2004a) measured adsorption of the pesticide dichlobenil by K^+- and Ca^{2+}-smectites from binary aqueous solutions of KCl and $CaCl_2$, and found that the adsorption increased with increasing molar ratio of K^+ to Ca^{2+}. At lower fractions of K^+ occupation of cation exchange sites, K^+ ions were randomly distributed on the external and interlayer clay surfaces and did not substantially enhance pesticide sorption. At higher populations of K^+ (vs. Ca^{2+}) demixing occurred causing some clay interlayers, interlayer regions or tactoids to become fully saturated with K^+ thereby manifesting greatly enhanced pesticide sorption. These results demonstrated that the degree of pesticide adsorption by smectite clays could be controlled by adjusting the amount of K^+ and Ca^{2+} associated with clay through simple geochemical cation exchange processes.

The objective of this study was to demonstrate the ability smectite clay to attenuate the bioavailability and hence toxicity of NACs to target organisms. There is a need to optimize bioavailability of contaminants during bioremediation so that they are sufficiently available for effective plant uptake or bioremediation without being present at levels that are toxic to the organisms used for biodegradation. The ability to modulate the binding and release, i.e. the bioavailability, of NACs via ion exchange processes on smectite clays is the focus of a subsequent study. The target organisms used in this study are two species of duckweed (*Lemna gibba and Lemna minor*) which are a group of free-floating aquatic plants often found growing on nutrient-rich water in ponds and lakes. Toxicity testing using duckweeds is an accepted and common protocol to evaluate aquatic toxicity of organic contaminants and heavy metals present in natural bodies of water (ASTM and Materials, 1991; Hillmans, 1961; Holst and Ellwanjer, 1982; Smith and Kwan, 1989; Taraldsen and Norberg-King, 1990; Wang and Freemark, 1995; Wu, 1980). The toxic effects of NACs to duckweed were evaluated to assess the bioavailability and toxicity of NACs in aqueous systems containing, and devoid of, K- smectite.

Materials and Methods

Chemicals

2,4-Dinitrotoluene (2,4-DNT) was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI) with a reported purity > 97%. Potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), potassium nitrate (KNO_3), potassium phosphate dibasic (KH_2PO_4), boric acid (H_3BO_3), sodium molybdate (Na_2MoO_4), sodium acetate (CH_3COONa) and cupric sulfate ($CuSO_4$) were purchased from Millinckrodt Baker Inc. (Phillipsburg, New Jersey); ferric chloride ($FeCl_3$), manganese chloride ($MnCl_2$), and zinc sulfate ($ZnSO_4$) were obtained from Sigma (St. Louis, MO); ethylenediaminetetracetic acid (EDTA) was obtained from Invitrogen Corporation (Carlsbad, CA.). All chemicals were used as received.

Clay preparation

Reference smectite clay (Wyoming montmorillonite, SWy-2) was obtained from the Source Clays Repository of the Clay Minerals Society (Purdue University, West Lafayette, Indiana) and used throughout this study. The SWy-2 clay has a CEC of 82cmol$_c$/kg and a theoretical surface area of 750m^2/g (van Olphen and Fripiat, 1979). The < 2μm clay particle-sized fraction was obtained by centrifuging a dilute clay suspension for 6 min at 60 *g* (~600rpm) using a Sorvall GSA rotor and RC-5C centrifuge (DuPont Co., Wilmington, Delaware) (Costanzo and Guggenheim, 2001). Prior to separating the clay-sized particles, the SWy-2 clay sample was treated with 0.5 M sodium acetate to remove carbonate impurities (Arroyo et al., 2005). The K-saturated homoionic clay was prepared using cation exchange reactions. Specifically, the naturally occurring inorganic exchangeable cations (predominately Na^+) on SWy-2 were replaced with K^+ by washing the clay-sized fraction of SWy-2 three times with 0.5M KCl solution. K-smectite clay suspensions were then dialyzed against Milli-Q water to remove excess electrolytes; the absence of white AgCl precipitate upon addition of $AgNO_3$ indicated that the clay suspensions were free of chloride. The prepared clay suspensions were quick-frozen and freeze-dried. Prior to use in phytotoxicity assays, K-SWy-2 clay was sterilized by heating at 120°C for 3-5 days.

Duckweed culture

Duckweeds were used as the phytotoxic indicator species for evaluating NAC exposure. *Lemna minor* and *Lemna gibba* duckweed cultures were obtained from the Culture Collection of Algae and Cyanobacteria (CCAC, University of Toronto; Toronto, Ontario, Canada). Axenic duckweed cultures were maintained in 300-mL Erlenmeyer flasks containing 150 mL of full-strength (1X) Hoagland's nutrient solution (Cowgill and Milazzo, 1989) at pH 5.8, supplemented with 10 g of sucrose per liter as recommended by the CCAC (Judy Acreman, personal communication). After autoclave sterilization of the sucrose-amended Hoagland's solution, 20 mL of a FeEDTA solution (prepared by dissolving 0.121 g $FeCl_3$ and 0.375g EDTA into 250-mL Milli-Q water and passed through a 0.22-μm filter) were added to complete the 1X duckweed medium (1X DWM). Culture flasks inoculated with duckweed were closed with sterilized foam plugs (~ 27 to 34 mm), the plugs covered with aluminum foil, and placed in a plant growth chamber (Boone, Iowa) at 25°C with a 16 hr day length and a photosynthetically active radiation (PAR) intensity of ~ 43μmol s^{-1} m^{-2}.

Plants were subcultured every 3 weeks by transferring approximately 16 fronds into a flask containing fresh sterile culture medium.

NAC phytotoxicity

Phytotoxic assays for 2,4-DNT were carried out using *Lemna minor* and *Lemna gibba* duckweed cultures in *in-vitro* bioreactors (described above). Using aseptic techniques in a laminar flow hood, 300 mg sterile K-SWy-2 clay were weighed into the 300 mL Erlenmeyer flasks, 150 mL of sterilized 1/5X DWM containing 7.5 mg/L 2,4-DNT and 0.01M KCl were added, and sterilized foam plugs partially inserted into the mouth of the flasks. Foam-plugged flasks were covered with aluminum foil to reduce the evaporative loss of water. The 1/5X DWM supplemented with 7.5 mg/L DNT and 0.01M KCl was sterilized by passing through a 0.2-μm membrane. Our preliminary study indicated that 150 mL of 1/5X DWM provided sufficient nutrients for duckweed growth and frond multiplication during the two week experimental period.

The effect clay sorption of 2,4-DNT on duckweed growth was evaluated as follows. Prior to inoculation with duckweeds, 150 mL of 1/5 X DWM containing 7.5mg/L 2,4-DNT and 0.01M KCl was mixed with 300 mg of K-SWy-2 clay in a 300-mL Erlenmeyer flask for four hours at room temperature to establish sorption equilibrium of 2,4-DNT with K-SWy-2 clay. To ascertain sorption equilibrium, the aqueous phase was periodically sampled and analyzed for 2,4-DNT concentrations by a high-performance liquid chromatography (HPLC). This study and a previous study (Sheng et al., 2002) have both indicated that sorption of NACs by K-smectites reached equilibrium within four hours.

To initiate the phytotoxicity assay, 8-10 green duckweed fronds were aseptically transferred to each flask. Foam plugs and foil were replaced and the flasks incubated without shaking in a growth chamber under the conditions described above. Controls consisted of the same 1/5X DWM without clay and/or without 2,4-DNT. Duckweed frond numbers were counted manually at selected sampling times as an index of NAC phytotoxicity. At pre-selected sampling times, 2 mL of solution were removed and analyzed for 2,4-DNT aqueous-phase concentrations using HPLC. In similar experiments, but in the absence of clay, 2,4-DNT toxicity to duckweed growth was measured over a range of 2,4-DNT concentrations from 0 to 20 mg/L in order to establish a dose-response relationship.

The HPLC system used to measure aqueous phase 2,4-DNT concentrations consisted of a Perkin-Elmer (Shelton, Connecticut) Binary 250 LC pump, a Series 200 autosampler and a Series 200 UV-visible detector. The mobile phase was an isocratic mixture of methanol and water (50:50) with the flow rate at 1.0 ml/min. The wavelength of the UV-visible detector was set at 250 nm.

Results and Discussion

The dose-response relationship of *Lemna gibba* and *Lemna minor* to aqueous-phase 2,4-DNT at different intervals of exposure is shown in Figure 1. The response of the two duckweed species, in terms of number of fronds produced, to 2,4-DNT solutions ranging from 0.5 to 20 mg/L (without clay) was evaluated to identify the level at which 2,4-DNT inhibits duckweed growth. The results indicated that *Lemna gibba* growth was negatively impacted to a measurable degree at 2,4-DNT concentrations ≥ 1.0 mg/L. *Lemna minor* was somewhat more susceptible to the toxicity of 2,4-DNT. For example, the observed difference in growth after 12 days exposure to 0.5 mg/L of 2,4-DNT compared with the 2,4-DNT-free controls was greater for *Lemna minor* compared to *Lemna gibba* (Figure 1).

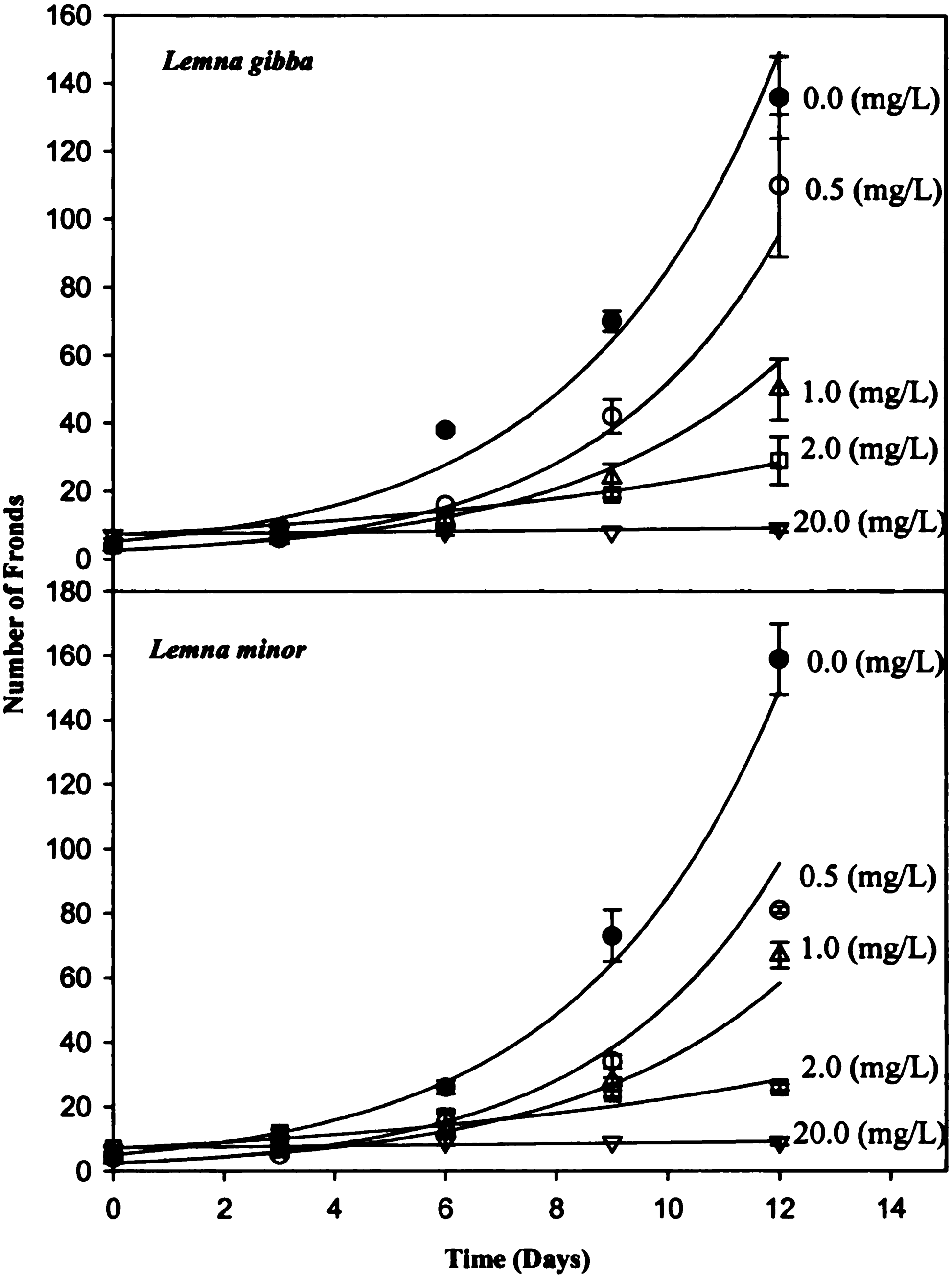

Figure 1. Growth of two species of duckweed over a 12 day incubation time as function of different solution concentrations (0 to 20 mg/L) of 2,4-dinitrotoluene.

To quantitate duckweed growth rate, the progress curves of ***Lemna minor*** and *Lemna gibba* plotted as frond number vs exposure period were fitted to a first-order kinetic process equation:

$$\ln F_t = \ln F_0 + kt \qquad [1]$$

where F_t and F_0 are the frond numbers at time t (days) and initial time, respectively, and k (day^{-1}) is the growth rate constant. The results of fittings are shown as solid lines in Figure 1. The growth data were well described by the equation [1]; fitting these data to the linear form of equation [1] gave r^2 values ≥ 0.95. Doubling time, T_2 (days $^{-1}$), of duckweed growth in terms of frond numbers was calculated using the equation:

$$T_2 = \ln 2 / k \qquad [2]$$

The growth rate constants and doubling times are listed in Table 1. For *Lemna minor* exposed to 2,4-DNT concentrations ≥ 0.5 mg/L, growth was inhibited as indicated by decreased growth rate constants and increased doubling time compared with the 2,4-DNT-free control. The doubling time increased approximately 10 and 20 % at 2,4-DNT concentrations of 0.5 and 1.0 mg/L, respectively, relative to that of the control when no 2,4-DNT is present. This corresponds to a > 50 % reduction in the number of fronds after 12 days of exposure to 2,4-DNT (Figure 1). As 2,4-DNT concentration increased to > 1.0 mg/L, toxic symptoms were usually apparent as the duckweed fronds changed from green to yellow-brownish in color. After 7 d exposure to 2,4-DNT ≥ 2.0 mg/L, duckweed fronds were white indicating the occurrence of severe chlorosis and death of the plants. No apparent growth was observed at 2,4-DNT concentration of 20 mg/L. The higher sensitivity of *Lemna* *minor* to 2,4-DNT exposure made it more suitable for use in our bioassays designed to test the effects of K-SWy-2 on 2,4-DNT toxicity and bioavailability (Figure 1 and Table1).

Table 1. Growth rate constant (k) and doubling time (T_2) of *Lemna minor and Lemna gibba* exposed to several levels of aqueous phase 2,4-dinitrotoluene (2,4-DNT) during 12 days of incubation.

	Lemna gibba			*Lemna minor*		
Aqueous 2,4-DNT Concentration (mg/L)	k (day $^{-1}$)	T_2 (days)	r^2 **	k (day $^{-1}$)	T_2 (days)	r^2
0	0.31	2.3	0.98	0.29	2.3	0.99
0.5	0.28	2.4	0.98	0.26	2.6	0.97
1.0	0.21	3.3	0.99	0.24	2.9	0.98
2.0	0.16	4.4	0.97	0.11	6.3	0.95
20.0	ng*			ng		

* no apparent growth; ** square of correlation coefficients from linear fitting of the first-order duckweed growth rate equation [1].

Among the factors influencing the bioavailability of organic contaminants in the soil environment, sorption is a major process which can reduce the bioavailability of organic compounds to target organisms. Nitroaromatic compounds have been reported to demonstrate strong affinities for K^+-saturated smectites (Boyd et al., 2001; Haderlein and Schwarzenbach, 1993; Haderlein et al., 1996; Johnston et al., 2001; Johnston et al., 2002; Li et al., 2004a; Sheng et al., 2002). As NACs are sorbed by K-smectites, their availability to aquatic plants may be reduced. The growth responses of *Lemna minor* to 7.5 mg/L of 2,4-DNT solutions in the presence and absence of K-SWy-2 clay after 11 d exposure are shown visually in Figure 2. In the system absent of K-SWy-2, the 7.5 mg/L 2,4-DNT solution completely inhibited *Lemna minor* growth as evidenced by failure to reproduce more fronds over the 11 d exposure period. The toxicity of 2,4-DNT at this concentration was apparent after 4 d exposure as duckweeds showed signs of chlorosis consisting of pale yellow and white spots on tissues. Death of *Lemna minor* occurred at ~ 9 d exposure. The addition of K-SWy-2 substantially reduced the inhibitory effects on frond production and toxicity arising from the presence of 7.5 mg/L of 2,4-DNT in the absence of clay. Addition of K-SWy-2 reduced 2,4-DNT concentration in water from 7.5 mg/L to 0.4 mg/L, an aqueous phase concentration which had minimal impact on duckweed growth in the absence of clay (Fig.1). In Figure 2, it is noted that duckweeds grew somewhat better in the system containing K-SWy-2 clay alone (without 2,4-DNT). Thus, the clay itself, independent of its ability to sequester 2,4-DNT, stimulated growth of *Lemna minor* to some extent.

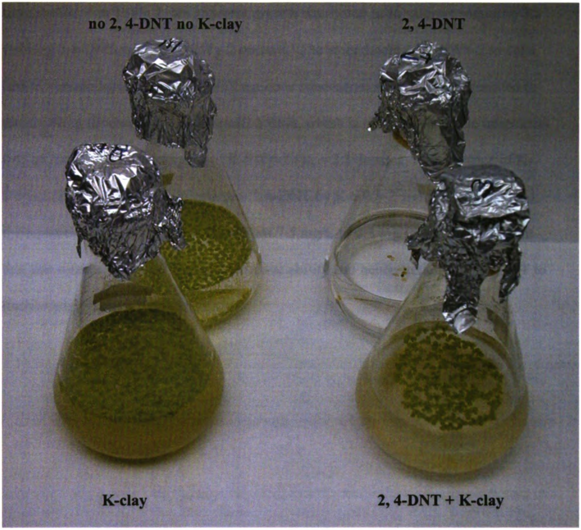

Figure 2. Photograph showing reduced bioavailability and toxicity of 2,4-dinitrotolune (2,4-DNT) to *Lemna minor* in the presence of homoionic K-montmorillonite (K-SWy-2) clay (300 mg clay in 150 mL solution). The photograph was taken after an incubation period of 11days. Treatments consisted of various combinations of 2,4-DNT and/or K-SWy-2.

The growth of *Lemna minor* in 1/5X DWM containing 7.5 mg/L 2,4-DNT in the presence and absence of K-SWy-2 as a function of exposure period is shown in Figure 3. The solid lines are fittings of the data to a first-order growth process described by equation [1], and the corresponding growth rate constants and doubling time are listed in Table 2. No apparent growth was noted in solutions containing 7.5 mg/L of 2,4-DNT without K-SWy-2 present. The amendment of K-SWy-2 to 1/5X DWM containing an initial 2,4-DNT aqueous concentration of 7.5 mg/L resulted in the doubling time of *Lemna minor* of 2.5 days, which is comparable to the treatments free of 2,4-DNT in the presence of K-SWy-2 (T_2 = 2.3 days) and the absence of K-SWy-2 (T_2 = 2.8 days). Adsorption of 2,4-DNT by K-SWy-2 significantly reduced the aqueous 2,4-DNT concentration (from 7.5 mg/L to 0.4 mg/L) and it is apparent that this reduction caused decreased bioavailability and hence toxicity of 2,4-DNT to duckweeds (Figures 2 and 3).

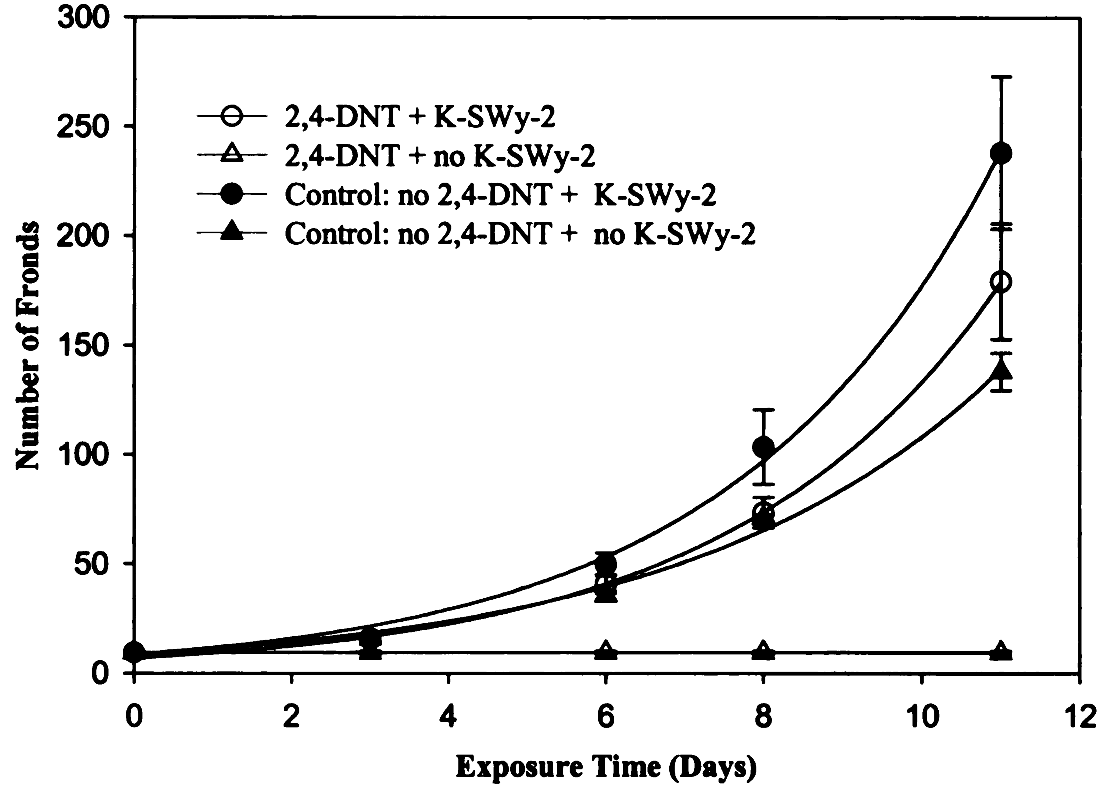

Figure 3. Effects of 2,4-dinitrotoluene (2,4-DNT) and clay on the growth rate of *Lemna minor* in 1/5X DWM over a incubation period of 11 days. Treatments were: 7.5 mg/L 2,4-DNT; 7.5 mg/L 2,4-DNT (added) plus homoionic K-montmorillonite (K-SWy-2); K-SWy-2; control containing neither 2,4-DNT nor K-SWy-2.

Table 2. Growth rate constant (k) and doubling time (T_2) of *Lemna minor* exposed to aqueous-phase 2,4-dinitrotoluene (2,4- DNT) in the presence and absence of K-smectite during an 11 day exposure period.

Treatments	Growth Rate Parameters		
	k (day $^{-1}$)	T_2 (days)	r^2 **
Free of K-SWy-2			
2,4-DNT (7.5 mg/L)	ng*	ng*	ng*
2,4-DNT (0.0 mg/L)	0.25	2.8	0.98
K-SWy-2 present			
2,4-DNT (7.5 mg/L)	0.27	2.5	0.99
2,4-DNT (0.0 mg/L)	0.30	2.3	0.97

* no apparent growth; ** square of correlation coefficients from linear fitting of growth to the first-order duckweed growth rate equation [1]

The results presented clearly indicate that sorption of 2,4-DNT by K-SWy-2 manifested a diminished bioavailability and toxicity to aquatic plants. This information could be useful in developing strategies for control of NAC mobility and bioavailability to target organisms such as plants and microorganisms utilized in *in-situ* bioremediation technologies. As part of a watershed geostabilization strategy, clay-modulated NAC sorption would allow sufficient phytoremediative plant biomass accumulation at otherwise phytotoxic soil contamination levels (e.g. of NACs). This 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 controlled fashion (Li et al., 2004b) into an active rhizosphere for effective plant uptake or bioremediation. This is the subject of an ongoing study in our laboratory.

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CHAPTER III

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

Abstract

Nitroaromatic compounds (NACs) are a prominent class of environmental toxic contaminants that pose a threat to human and ecosystem health. Nitroaromatic compounds demonstrate a high affinity with smectite clays which are widely distributed in nature as components of soils and sediments, and in geological deposits. The magnitude of NAC sorption by smectites is highly dependent on the exchangeable cation. For example, smectites saturated with K^+ have a very high affinity for NACs while those saturated with Ca^{2+} do not. In this study the ability of K^+-saturated smectite to attenuate the bioavailability and hence toxicity of 2,4-dinitrotoluene (2,4-DNT) to the aquatic plant duckweeds (*Lemna gibba* and *Lemna minor*) was evaluated. 2,4-DNT at the concentration of 7.5 mg/L was highly toxic to duckweeds. However, the presence of small amounts of K-smectite in aqueous growth media reduced this toxicity substantially because the sorption of 2,4-DNT by the added smectite reduced bioavailability. This reduced bioavailability could be modulated by simple K^+/Ca^{2+} cation exchange reaction occurring on mineral surfaces. In the subsequent experiments, $CaCl_2$ was added and the Ca^{2+} replaced K^+ associated with smectite surfaces. Such reaction released clay-sorbed NACs to aqueous solution, which is readily available to target plants. This study proves the concept that we can modulate the type and fraction of cations occupying the cation-exchange-capacity sites of smectite clays to control the sorption/desorption hence bioavailability of NACs in smectite-rich soils and sediments. This information is needed in developing strategies for control of NAC mobility and bioavailability to

target organisms such as plants and microorganisms utilized in *in-situ* bioremediation technologies.

Introduction

Smectites are expandable 2:1 layered alumina silicate clays that are widely distributed in nature (Allen and Hajek, 1989). They have structural negative charges arising from isomorphic substitution in the octahedral and/or tetrahedral layers. In nature, common inorganic cations (e.g., Ca^{2+}, Mg^{2+}, Al^{3+}, K^{+}, and Na^{+}) exist on clay surfaces to balance the negative charges. These exchangeable cations control clay interlayer expansion via hydration, and may interact with environmental contaminants/ pesticides, thereby leading to sorption from water and immobilization. Among the clay minerals commonly found in soils, smectites may function as particularly effective adsorbents for organic contaminants and pesticides because of their high surface areas, surface acidity and reactivity, large cation exchange capacities, and reversible interlayer expansibility.

Sorption by soils/sediments is a major determinant of environmental fate of organic contaminants and pesticides, such as potential for leaching and bioavailability to microorganisms and plants (Koskinen and Harper, 1990). Sorption often renders contaminants less available to receptor organisms (Hatzinger and Alexander, 1995; Luthy et al., 1997; Ogram et al., 1985; Pignatello and Xing, 1996). Scribner et al., (1992) and Steinberg et al., (1987) showed that ethylene dibromide and simazine sorption and sequestration by field weathered soils reduced bioavailability to contaminant-biodegrading microorganisms thereby prolonging residence times. Reduced bioavailability in these instances was associated with prolonged pesticide-soil contact times. A number of studies have examined the bioavailability of sorbed contaminants to microorganisms (Boyd et al., 1992; Feng et al., 2000; Park et al., 2004). There are examples where bacteria can and cannot access contaminants sorbed to soils. At this point, no generalizations can be made regarding the bioavailability of sorbed organic contaminants. Harris (1964) showed lowered bioavailability of insecticides (to crickets) sorbed to dry soils as evidenced by higher LD_{50} values compared to the insecticides in the non-sorbed state. Wetting the soils apparently caused desorption of the insecticides, as LD_{50} values decreased by an order of magnitude or more. Soil minerals in the dry state have been shown to sorb much higher amounts of organic contaminants than occurs in the presence of water (Chiou 2002). It was hypothesized that water molecules preferentially occupied the mineral surfaces rendering the ability of soil mineral to sorb and retain organic molecules in the presence of water. Recent work on the bioavailability of 2,4-dinitrotoluene (2,4-DNT) showed that in water it was highly toxic to the aquatic duckweed (Roberts et al., 2006). However, sorption of 2,4-DNT by K-saturated smectite in aqueous media reduced its bioavailability and hence toxicity to duckweed.

Nitroaromatic compounds (NACs) are components of munitions that are commonly found as soil and ground water contaminants in military training facilities and manufacturing sites. For example, 10% of the NACs in many munitions may fall to the soil as unaltered NAC after an ordinance explodes (Taylor et al., 2004). Nitroaromatic compounds also include important pesticides such as the dinitrophenol herbicides that may contaminate ground- and surface-waters or persist in soils where they are used in crop protection (Broholm, 2001; Hawley, 1981). As such, NACs pose threats to human and ecosystem health (Berthe-Corti et al., 1998; Gong et al., 1999; Hawley, 1981; Neumann et al., 1995). For instance, the $-NO_2$ of NACs in organisms can be reduced to amino groups that conjugate with the phenolic sites in biota (Morgan, 1989; Ware, 1997), with toxic effects (Gong et al., 1999; Sunahara et al., 1998).

Many smectites demonstrate high affinity for aqueous phase NACs, and sorption is strongly influenced by the type of cations on ion-exchange sites of the clays. For example, smectites exchanged with Cs^+, K^+ and NH_4^+ have high affinities for NACs whereas smectites display much lower NAC adsorption when exchanged with Na^+, Ca^{2+} or Mg^{2+} (Boyd et al., 2001; Haderlein and Schwarzenbach, 1993; Haderlein et al., 1996; Johnston and Premachandra, 2001; Johnston et al., 2002; Li et al., 2004a; Sheng et al., 2002). Strong hydration of Na^+, Ca^{2+} and Mg^{2+} cations creates an unfavorable clay interlayer environment for NAC adsorption, whereas the comparatively weak hydration of Cs^+, K^+ and NH_4^+ cations creates a much more favorable interlayer environment for NAC adsorption. Adsorption of NACs occurs primary in the clay interlayer and weaker cation hydration manifests a more optimal interlayer distance, larger adsorptive domains, enhanced ability to form NAC-cation complexes, and greater solute dehydration (Boyd et al., 2001; Johnston and Premachandra, 2001; Johnston et al., 2002; Li et al., 2004a; Sheng et al., 2002).

Among the factors influencing the bioavailability of organic contaminants in the soil environment, sorption is a major process that can reduce the bioavailability of organic compounds to target organisms. In the case of NACs, smectite clay surface chemistry can be used to control sorption and bioavailability of NACs by simple ion-exchange reactions that change the interlayer ion composition of the clays. For example, a K^+-saturated system would maximize NAC retention and could be used to minimize transport of the environmental contaminants. This was indicated in a recent study where KCl solutions injected into an aquifer were shown to reduce transport of nitrotoluene, and subsequent injections of $CaCl_2$ were shown to remobilize nitrotoluene (Weissmahr et al., 1999). In another study, K-smectite was used to promote aqueous phase NAC adsorption, thereby reducing bioavailability and toxicity of the NAC to aquatic plants (Roberts et al., 2006). Li et al., (2004a) measured adsorption of the pesticide dichlobenil by mixed K^+/Ca^{2+}-smectites and found that the adsorption increased with increasing molar ratio of K^+ to Ca^{2+}on the cation exchange sites of montmorillonite. Their results demonstrated that the degree of pesticide adsorption by smectite clays could be controlled by adjusting the amount of K^+ and Ca^{2+} associated with clay through simple geochemical cation exchange processes. Thus, simple reactions such as Ca-for-K ion exchange might hold promise as a method to modulate the bioavailabilities of NACs in soils and sediments.

The objective of this study was to demonstrate the ability to modulate the bioavailability and toxicity of NACs by controlling their binding and release via cation ion exchange reactions on smectite clays. There is a need to optimize bioavailability of contaminants during bioremediation so that they are sufficiently available for effective plant uptake or bioremediation without being present at levels that are toxic to the organisms used for biodegradation. The target organism used in this study was duckweed (*Lemna minor*), a group of free-floating aquatic plants usually found growing on nutrient-rich water in ponds and lakes. Toxicity testing using duckweeds is an accepted and common protocol for evaluating the aquatic toxicities of organic and heavy metal contaminants (ASTM and Materials, 1991; Hillmans, 1961; Holst and Ellwanjer, 1982; Smith and Kwan, 1989; Taraldsen and Norberg-King, 1990; Wang and Freemark, 1995; Wu, 1980).

Materials and Methods

Chemicals

2,4-Dinitrotoluene (2,4-DNT) was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI) with a reported purity > 97%. Potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$), potassium nitrate (KNO_3), potassium phosphate dibasic (KH_2PO_4), boric acid (H_3BO_3), sodium molybdate (Na_2MoO_4), sodium acetate (CH_3COONa) and cupric sulfate ($CuSO_4$) were purchased from Mallinckrodt Baker Inc. (Phillipsburg, New Jersey). Ferric chloride ($FeCl_3$), manganese chloride ($MnCl_2$), and zinc sulfate ($ZnSO_4$) were obtained from Sigma (St. Louis, MO), and ethylenediaminetetracetic acid (EDTA) was obtained from Invitrogen Corporation (Carlsbad, CA.). All chemicals were used as received.

Clay preparation

Reference smectite clay (Wyoming montmorillonite, SWy-2) was obtained from the Source Clays Repository of the Clay Minerals Society (Purdue University, West Lafayette, Indiana) and used throughout this study. The SWy-2 clay has a CEC of 82 $cmol_c/kg$ and a theoretical surface area of 750 m^2/g (van Olphen and Fripiat, 1979). The < 2μm clay particle-sized fraction was obtained by centrifuging a dilute clay suspension for 6 min at 60 *g* (~600rpm) using a Sorvall GSA rotor and RC-5C centrifuge (DuPont Co., Wilmington, Delaware) (Costanzo and Guggenheim, 2001). Prior to separating the clay-sized particles, the SWy-2 clay sample was treated with 0.5 M sodium acetate to remove carbonate impurities (Arroyo et al., 2005). The K-saturated homoionic clay was prepared using cation exchange reactions. Specifically, the naturally occurring inorganic exchangeable cations (predominately Na^+) on SWy-2 were replaced with K^+ by washing the clay-sized fraction of smectite three times with 0.5 M KCl solution. K-smectite suspensions were then dialyzed against Milli-Q water to remove excess electrolytes; the absence of white AgCl precipitate upon addition of $AgNO_3$ indicated that the clay suspensions were free of chloride. The prepared clay suspensions were quick-frozen and freeze-dried. Prior to use in phytotoxicity assays, K-smectite clay was sterilized by heating at 120°C for 3-5 days.

Duckweed culture

Duckweeds were used as the phytotoxic indicator species for evaluating NAC exposure. *Lemna minor* and *Lemna gibba* duckweed cultures were obtained from the Culture Collection of Algae and Cyanobacteria (CCAC, University of Toronto; Toronto, Ontario, Canada). Axenic duckweed cultures were maintained in 300-mL Erlenmeyer flasks containing 150 mL of full-strength (1X) Hoagland's nutrient solution (Cowgill and Milazzo, 1989) at pH 5.8, supplemented with 10 g of sucrose per liter as recommended by the CCAC (Judy Acreman, personal communication). After autoclave sterilization of the sucrose-amended Hoagland's solution, 20 mL of a FeEDTA solution (prepared by dissolving 0.121 g $FeCl_3$ and 0.375g EDTA into 250-mL Milli-Q water and passed through a 0.22-μm filter) were added to complete the 1X duckweed medium (1X DWM). Culture flasks inoculated with duckweed were closed with sterilized foam plugs (~ 27 to 34 mm), the plugs covered with aluminum foil, and placed in a plant growth chamber (Boone, Iowa) at 25°C with a 16 hr day length and a photosynthetically active radiation (PAR) intensity of ~43 $\mu mol\ s^{-1}\ m^{-2}$.

Plants were subcultured every 3 weeks by transferring approximately 16 fronds into a flask containing fresh sterile culture medium.

Sorption Experiments

Sorption of 2,4-DNT by K-SWy-2 from KCl or $CaCl_2$ solutions was measured using a batch equilibration technique. Clay was weighed into triplicate glass centrifuge tubes, aqueous solutions over a range of initial 2,4-DNT concentrations were added, and the tubes were closed immediately with Teflon-lined screw caps. The tubes were shaken end-over-end at 40 rpm for 24 h at room temperature. The tubes were centrifuged for 30 min to separate liquid and solid phases. Previous studies demonstrated that sorptive equilibrium has been reached within 4 h (Sheng et al., 2002). Concentrations of NACs in supernatants were measured by a Perkin-Elmer High-Performance Liquid Chromatography (HPLC) system that consisted of a Binary 250 LC pump, a Series 200 autosampler and a Series 200 UV-visible detector. The optimal wavelength was set at 250 nm for 2,4-DNT. The mobile phase composition was a 1:1 mixture of methanol and water. Control samples included the initial aqueous 2,4-DNT solutions in the absence of clay at each 2,4-DNT concentration. No changes in solute concentrations were detected in the tubes containing aqueous 2,4-DNT without clay; therefore, solute mass lost from the supernatant in the presence of clay was assumed to be sorbed by clay. The sorbed concentrations were calculated from the difference between control and final solute mass in aqueous solution. Sorption isotherms were obtained by plotting the amount of sorbed by clay against the equilibrium aqueous phase concentrations.

NAC phytotoxicity

Phytotoxic assays for 2,4-DNT were carried out using *Lemna minor* duckweed cultures in *in-vitro* bioreactors containing duckweed culture medium with a 1/5-strength of Hoagland's solution (1/5X DWM). Using aseptic techniques in a laminar flow hood, 300-mg sterile K-SWy-2 clay were weighed into 300-mL Erlenmeyer flasks, 75 mL of 1/5X DWM containing 7.5 mg/L 2,4-DNT added, and sterilized foam plugs partially inserted into the mouth of the flasks. Foam-plugged flasks were covered with aluminum foil to reduce the evaporative loss of water.

The effects clay sorption of 2,4-DNT on duckweed growth was evaluated as follows. Prior to inoculation with duckweed, 75 mL of 2,4-DNT solution was added to flasks that contained 300 mg of sterilized K-SWy-2 clay and then the flasks were shaken for four hours at room temperature to establish sorption equilibrium of 2,4-DNT with K-smectite. This volume of solution (75 mL) provided sufficient surface area and volume for duckweed growth and multiplication. To ascertain sorption equilibrium, the aqueous phase was periodically sampled and analyzed for 2,4-DNT concentrations using HPLC. This study and previous studies both indicated that sorption equilibrium of NACs by K-smectites was reached within four hours (Sheng et al., 2002).

To initiate the phytotoxicity assay, 8-10 green healthy fronds were aseptically transferred to each flask. The foam plugs and foil were replaced and the flasks incubated in a growth chamber under the conditions described previously. Thus, the bioavailability bioassays consisted of 75 ml of 1/5-strength Hoagland solution containing 0.01M KCl background electrolyte, 7.5 mg/L 2,4-DNT, 300 mg of K-

SWy-2 clay and 8-10 fronds. Controls consisted of the same 1/5X DWM with clay but without 2,4-DNT. Duckweed frond numbers were counted manually at selected sampling times up to 408 hours of incubation as a measure of NAC phytotoxicity. At pre-selected sampling time (at 0, 4, 9, 11 and 17 days) 2 mL of solution were sampled, transferred to vials and then the 2,4-DNT aqueous-phase concentrations were measured using HPLC.

Release of 2,4-DNT into solution

After four days of incubation and upon the establishment of 20 duckweed fronds, 75 mL of either 0.1 M $CaCl_2$ or 0.01 M KCl (both in 1/5 DWM) were added to the flasks to evaluate the release or retention of sorbed-2,4-DNT by the smectite clay and the consequent effects on duckweed growth. These bioreactor flasks with a final volume of 150 mL were shaken for four hours to mix the systems and then incubated in a growth chamber under the same conditions described previously. Controls consisted of the same solution treatments with duckweeds and clay, but without 2,4-DNT.

A similar bioassay as was used to isolate the effects of cations themselves on duckweed growth in the absence of clay and NAC. That is, after four days of incubation, 75 mL of 0 to 0.2 mol/L Cl solutions of K^+, NH_4^+, or Ca^{2+} in 1/5 DWM were added to the bioreactors, and subsequent duckweed growth was monitored.

Also, duckweed biomass, as an alternative index for duckweed growth was measured using the following procedure. Duckweed fronds were removed from flasks, rinsed four times with distilled water, blotted with tissue paper, and the whole plants (fronds and roots) immediately weighed to obtain the fresh weight. The tissues were then dried in an oven at 70 °C until each sample achieved a constant weight (dried biomass). The mean of four samples at each sampling interval was considered one observation. Four replicate samples were sacrificed for measuring biomass at 0, 4, 9, 11 and 17 days of incubation.

A completely randomized design was adopted. Before comparing treatment differences, the natural log transformation was applied because we observed trends in treatments of the original data which had larger variance at later time points compared to the small variance of early time points. The analysis of variance (ANOVA) was applied to determine the statistical significance ($P<0.05$) for differences in frond numbers among treatments. Statistical analyses were performed on data from exposure day 4 to day 17 using mixed procedures (PROC MIXED; SAS Inst., 2005) to analyze treatment differences in duckweed frond numbers.

Results and Discussion

Smectite clay is an effective sorbent for 2,4-DNT, with the extent of sorption depending largely on the exchangeable cations occupying the CEC sites of the clay. Sorption of 2,4-DNT from 0.01 M KCl and 0.005 M $CaCl_2$ by K-smectite is represented in Figure 1 as sorption isotherms, with amount sorbed per unit mass of clay plotted against equilibrium aqueous concentration. Sorption of 2,4-DNT by K-smectite was substantially reduced in the presence of 0.005 M $CaCl_2$ compared to that in KCl solution. For example, at aqueous 2,4-DNT concentration of 2.0 mg/L, sorption of 2,4-DNT in 0.005 M $CaCl_2$ was about 1/10 the sorption in 0.01 M KCl solution. The lowered affinity of 2,4-DNT for K- smectite is attributed to ion exchange of K^+ on K-smectite by Ca^{2+}. Specifically, in 0.005M $CaCl_2$, Ca^{2+}replaces some K^+ on smectite eliminating some K^+-saturated clay domains/interlayers where NAC sorption is believed to occur (Li et al., 2004b).The favorable clay-NAC interactions afforded by K- smectite such as the formation of complexes between the $-NO_2$ groups and K^+ associated with the clay and/or electron-donor-and-acceptor interactions between clay surfaces and planar aromatic rings of NACs, are diminished with Ca^{2+} as the exchangeable cation (Boyd et al., 2001; Haderlein and Schwarzenbach, 1993; Haderlein et al., 1996; Li et al., 2004b). The relatively strong hydration of Ca^{2+} results in a larger hydration sphere surrounding it thereby, inhibiting complex formation with NACs and interactions with clay surfaces (Boyd et al., 2001; Haderlein et al., 1996). Furthermore, Ca^{2+} on exchange sites of smectites causes greater interlayer swelling compared to K-saturated smectites (Boyd et al., 2001; Haderlein et al., 1996; Johnston and Premachandra, 2001; Johnston et al., 2002;

Sheng et al., 2002), which may also reduce hydrophobic contributions to NAC sorption (Boyd et al., 2001; Li et al., 2004b). As a consequence, addition of Ca^{2+} caused 2,4-DNT sorption by K-smectite to be reduced. We propose that such a cation exchange process can be utilized to control the retention/release of 2,4-DNT from clay hence altering the bioavailability and toxicity of 2,4-DNT to target organisms.

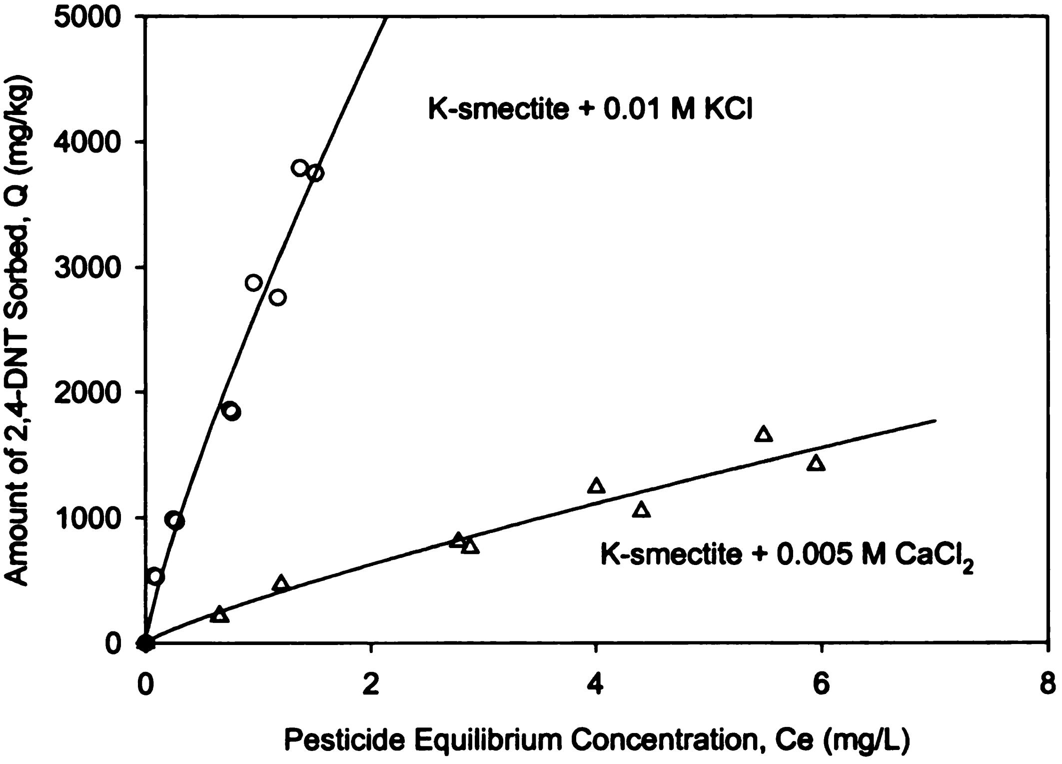

Figure 1. Adsorption of 2,4-DNT by K-smectite suspensions in presence of 0.01M KCl or 0.005 M $CaCl_2$.

Response of Lemna minor duckweed to added K^+, NH_4^+ and Ca^{2+}

Lemna duckweed is clearly sensitive to the toxic effect of 2,4-DNT, but for subsequent assays in the presence of clay, it was important to distinguish between the toxicity of the test compound and possible effects emanating from cations associated with the smectite clay surfaces. The cations K^+, NH_4^+ and Ca^{2+} at aqueous concentrations between 0 and 0.2 M were tested for their effect on growth of *Lemna* duckweeds (Fig.2). These cations are candidates for modulating the binding and release of NACs by clays (Li et al., 2004b) and hence provide the basis for controlling bioavailability and toxicity of NACs. Smectite clays saturated with K^+ or $NH_4^+$are strong sorbents for NACs whereas Ca-smectite is much less effective in removing NACs from aqueous solution (Boyd et al., 2001). Thus, by controlling the type and distribution of exchangeable cations on smectite clays, NACs may be selectively removed from water or released into solution (Li et al., 2004b). In Figure 2, the *Lemna minor* response to the cations at concentrations ranging between 0.01 and 0.2 M for K^+, NH_4^+, and Ca^{2+}in solution are compared to controls without added exchangeable cations. Evaluating the ability of plants to grow in the presence of these cations (in absence of clay) is necessary to determine which cations, at what concentrations, might cause toxicity thereby complicating our assay for evaluating the effectiveness of smectites for controlling toxicity and availability of NACs duckweeds. Even at the comparatively low aqueous concentration of 0.01M, NH_4^+ substantially reduced the number of fronds. At 0.1 M, NH_4^+, K^+ and Ca^{2+} all substantially limited the number of fronds produced. Thus, NH_4^+ at all concentrations tested, and higher concentrations of K^+ and Ca^{2+} ($\geq$ 0.05 M) were found to inhibit plant growth. Since 0.01 M of KCl had no toxic effect on plant growth, it was chosen as a background electrolyte solution in our bioassays. K^+-smectite clay was selected as the model adsorbent in this study since it is known to be an effective adsorbent of many NACs including 2,4-DNT. A background solution of 0.01 M K^+ ensures that small amounts of K^+ in bulk solution, due to the presence of K-smectite, will not contribute to the apparent toxicity of 2,4-DNT to duckweeds used in our bioassays.

Table 1. Growth rates (k) and doubling times (T_2) of *Lemna minor* during 12 day incubations in 1/5 X DWM containing added cations K^+ and Ca^{2+} at different molar concentrations. Doubling time were calculated using: T_2 (days) = (ln (2)/k).

Treatments	Added Cation-K^+			Added Cation-Ca^{2+}		
Concentrations of added cations (M)	k (day^{-1})	T_2 (days)	r^2	k (day^{-1})	T_2 (days)	r^2
No added cations	0.26	2.7	0.99	0.26	2.7	0.99
0.01	0.28	2.5	0.98	0.29	2.4	0.97
0.05	0.21	3.3	0.99	0.20	3.5	0.99
0.1	0.12	5.6	0.95	0.09	7.1	0.99
0.2	* ng	ng	ng	0.01	46.5	0.5

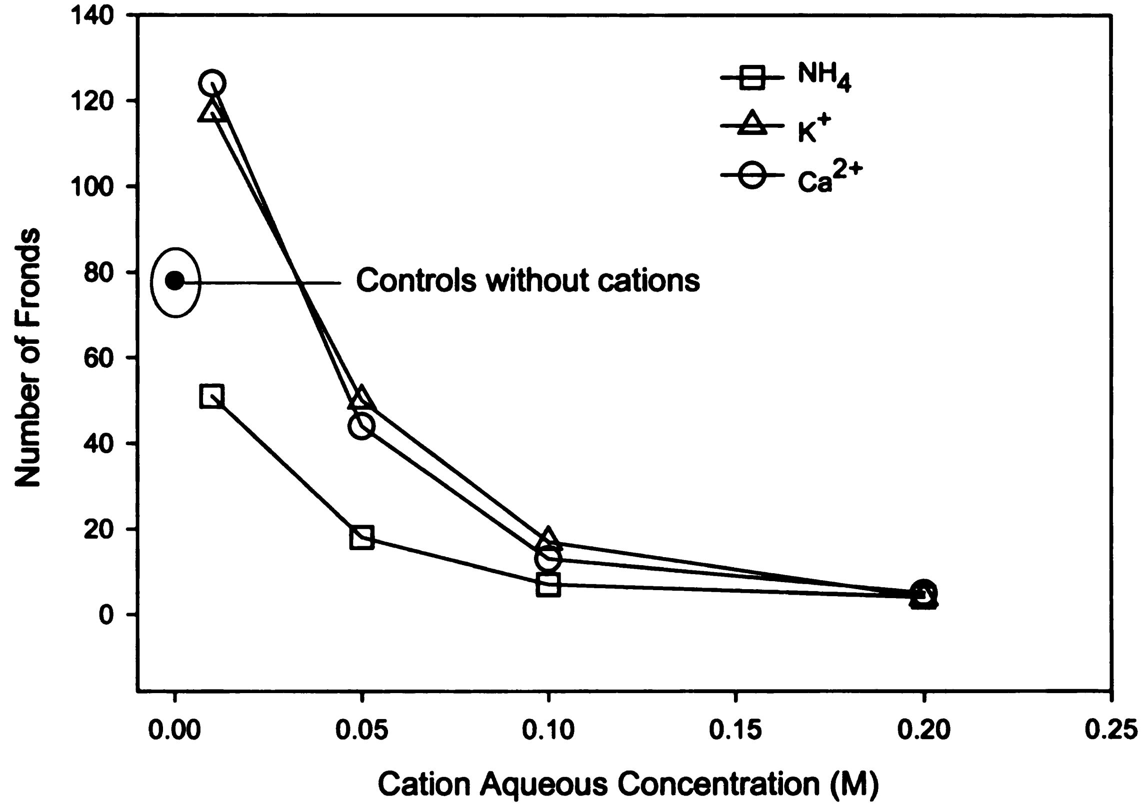

Figure 2. Growth of duckweed after 12 day bioassay in presence of different electrolyte solutions at concentrations ranging from 0.01 and 0.20M. The electrolytes chosen contain cations that might be used to control binding (NH_4^+, K^+) and release (Ca^{2+}) of nitroaromatic compounds by smectite clay.

To test the hypothesis that K^+/Ca^{2+} exchange reactions can be used to modulate the bioavailability of 2,4-DNT to a target organism (e.g. plants and microbes), the duckweed *Lemna minor* was identified as an appropriate plant for this study (Roberts et al., 2006). Figure 3 shows the responses of *Lemna minor* to the presence of 2,4-DNT in K-SWy-2 suspensions in terms of frond numbers and biomass production. The 2,4-DNT aqueous concentration was also monitored during the 17-day incubation. At the initiation of the experiment K-smectite (300mg) was added which substantially reduced the 2,4-DNT concentration in water from 7.5 mg/L to < 0.31 mg/L, an aqueous phase concentration that has minimal impact on *Lemna minor* growth (Roberts et al., 2006). This is seen during the first 4-d exposure, where there were no differences in duckweed growth in the K-smectite suspensions with the added 2,4-DNT and the 2,4-DNT-free controls (Figure 3). Figures 4a and 4b visually show that there were no observable differences after 4 days of exposure.

It was necessary to determine growth conditions in our bioassays that would allow us to distinguish the potential effects of Ca^{2+}/K^{+} ion exchange on the release of 2,4-DNT into solution and subsequent effects on bioavailability and toxicity to duckweed.

At 4-d exposure, since there were a sufficient number of fronds present (but not too many) and no apparent difference in the number of fronds produced between the two systems (2,4-DNT and 2,4-DNT-free controls), electrolyte solutions of KCl and $CaCl_2$ were added to initiate the ion exchange process. Such addition of electrolytes at 4 d of exposure was appropriate for visualizing of the effects of toxicity of 2,4-DNT. In our previous efforts developing the appropriate bioassays conditions there was no apparent inhibition on growth of *Lemna minor* at the 1/5X DMW concentration used, and the 150 mL volume of solution provided sufficient surface area of water for plants to grow and be distinguishable so that frond counts be made during a two week incubation period.

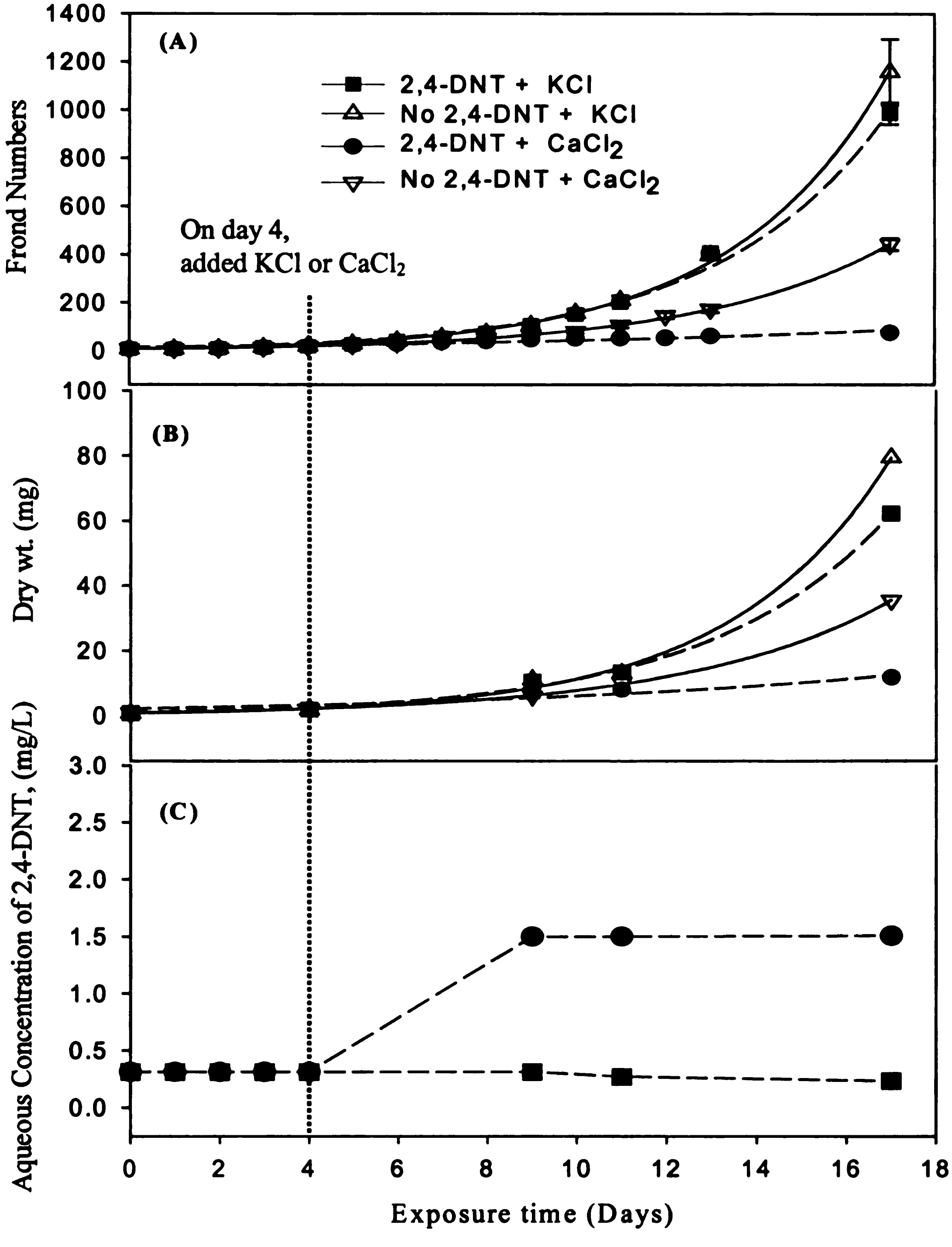

Figure 3. The growth of *Lemna minor* in the presence of 2,4-dinitrotoluene (2,4-DNT) and K-smectite as affected by addition (day 4) of KCl or $CaCl_2$ solutions. Growth and toxicity are indicated by frond numbers (A) and biomass (B). Aqueous concentrations of 2,4-DNT during the 17 day growth experiment are show in panel (C).

At day 4, 0.1M $CaCl_2$ or 0.01M KCl were added into the clay-duckweed systems with or without 2,4-DNT. *Lemna minor* growth curves were nearly identical for KCl-added systems whether 2,4-DNT was present or not (Figure 3A). In the absence of 2,4-DNT, the addition of $CaCl_2$ slowed down frond production compared to that with KCl addition. In the suspensions containing 2,4-DNT, the addition of $CaCl_2$ caused the aqueous concentration of 2,4-DNT to increase (Fig. 3C) resulting in inhibition of duckweed growth. Therefore, in the case of adding 0.1M $CaCl_2$ to 2,4-DNT-clay system, itself apparently caused some inhibitory effects on duckweed growth, consistent with results shown in Fig. 2, however the inhibitory effects if Ca^{2+} vs. 2,4-DNT could be easily discerned. In summary, the 2,4-DNT released into solution as a result of $CaCl_2$ addition (Fig. 3C) caused complete cessation of *Lemna minor* growth, clearly demonstrating that 2,4-DNT became available hence toxic to the duckweeds. We made comparisons of frond numbers (means of four replicates) at different periods of exposure to determine at which point toxicity effects due to release of 2,4-DNT associated with $CaCl_2$ addition were most apparent (Table 3). Between days 9 and 11, toxic symptoms were most obvious. This corresponded to visual observation that by day 11 the *Lemna minor* fronds had changed from green to yellow-brownish in color. After 17 days, in the systems with $CaCl_2$ and 2,4-DNT, fronds turned white indicating the occurrence of severe chlorosis and death of the plants. The measured dried biomass (Figure 3B) also showed the same trend as that revealed by frond numbers.

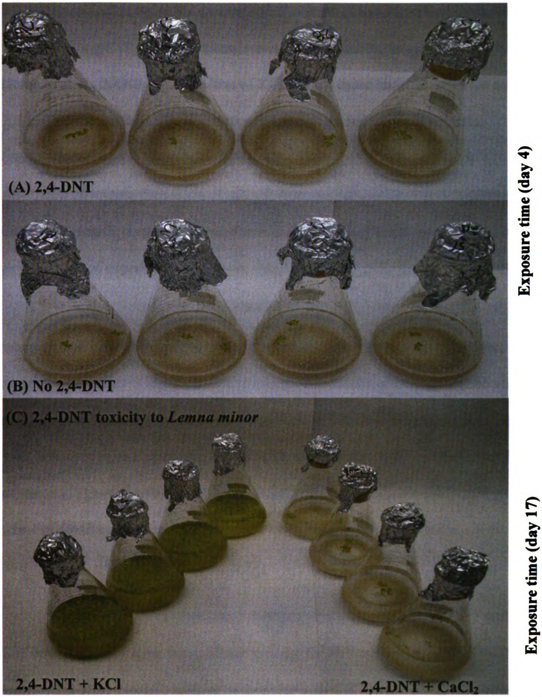

Figure 4. The effects of 2,4-dinitrotoluene (2,4-DNT) in K-smectite suspensions on duckweed (*Lemna minor*) growth. Duckweed frond numbers were equal in presence (A) or absence (B) of 2,4-DNT on day 4 when either KCl or $CaCl_2$ solution were added. The effects of these treatments after 17 day incubations are shown in panel (C). Addition of $CaCl_2$ caused release of 2,4-DNT into solution where it inhibited growth.

The addition of $CaCl_2$ to K-smectite suspension containing (mostly sorbed) 2,4-DNT resulted in 2,4-DNT concentration in water phase increasing from 0.31 mg/L to 1.5 mg/L (Figure 3C), a concentration that inhibits the growth of duckweeds (Roberts et al., 2006)). The addition of KCl did not cause an increase in the aqueous 2,4-DNT concentration. In fact, it resulted in a slight reduction of aqueous concentration (from 0.31 to 0.23 mg/L).

To quantitate the effects of salt solutions and of 2,4-DNT toxicity on duckweed growth, progress curves showing *Lemna minor* frond numbers and biomass as a function of exposure periods were fitted to the first-order kinetic processes.

$$\ln F_t = \ln F_0 + kt \qquad [1]$$

where F_t and F_0 are the frond number or biomass at the time t and initial time, respectively, k (day^{-1}) is growth rate constant. The results of fittings are shown as solid lines in Figures 2, 3A and 3B. The effects of 2,4-DNT on growth data were well described by the equation [1]; fitting the frond numbers data to the linear form of equation [1] gave r^2 values ≥ 0.95. Doubling times, T_2 (days) for duckweed growth in terms of frond number and biomass were calculated using the equation:

$$T_2 = \ln 2 / k \qquad [2]$$

The growth rate constants and doubling times are listed in Tables 1 and 2. The doubling times were lower (faster growth) for KCl treatments (with 2,4-DNT and with no 2,4-DNT present) relative to those for both $CaCl_2$ treatments, indicating the different degrees of bioavailability and toxicity of 2,4-DNT to duckweeds over the course of incubation. In the key experiment, i.e. the addition of $CaCl_2$ to K-smectite systems containing (mostly sorbed) 2,4-DNT, the effects of 2,4-DNT release (induced by Ca^{2+} exchange for K^{+}) on growth was evidenced by substantially decreased growth rate constants and increased doubling times compared with the effect of $CaCl_2$ addition when no 2,4-DNT is present, and to all other systems evaluated. The doubling time (which expresses the level of 2,4-DNT toxicity) for the K-smectite/$CaCl_2$ system with 2,4-DNT present was 5 days compared to 1.9 days for the K-smectite/KCl system with 2,4-DNT present (Table 2). In the K-smectite/$CaCl_2$ system containing 2,4-DNT, the doubling times increased approximately 163 and 100 based on frond production and biomass, respectively, relative to the same system amended with KCl rather than $CaCl_2$.

Table 2. Growth rate (k) and doubling times (T_2) of duckweed *Lemna minor* during 17 day incubations; values derived from measurements of fronds and biomass produced. Doubling time (days) calculated using: T_2 (days) = (ln (2)/k). Treatments on day 4 consisted of 0.01M KCl or 0.1M $CaCl_2$ added to K-smectite suspensions containing, 2,4-DNT and equal number of fronds. Controls were identical but did not contain 2,4-DNT. Addition of Ca^{2+} caused release of sorbed 2,4-DNT into solution resulting in decreased frond numbers and biomass.

	Frond production			Biomass production		
Treatments	k (day^{-1})	T_2 (days)	r^2	k (day^{-1})	T_2 (days)	r^2
Control: no-DNT + KCl	0.35	2.0	0.99	0.28	2.4	0.99
Control: no-DNT + $CaCl_2$	0.25	2.7	0.98	0.22	3.1	0.99
2,4-DNT + KCl	0.37	1.9	0.99	0.27	2.6	0.98
2,4-DNT + $CaCl_2$	0.14	5.0	0.95	0.13	5.2	0.86

Comparisons were made among the treatments with 2,4-DNT and controls without 2,4-DNT at a given exposure time to determine which days 2,4-DNT toxicity to duckweed became apparent. The treatment effects are easy to discern (Table 3). At day 4, when systems were amended with KCl or $CaCl_2$, there were no significant differences in frond numbers among treatments. At day 7, the KCl amended systems frond numbers with and without 2,4-DNT, were not statistically different. Frond numbers in the $CaCl_2$ amended system with 2,4-DNT were significantly lower than in the KCl plus 2,4-DNT system and in the $CaCl_2$ system without 2,4-DNT. At subsequent times differences between the $CaCl_2$ plus 2,4-DNT and KCl plus 2,4-DNT treatments remained significant, with the later having higher frond numbers. By day 17, the differences in these two treatments were very large; 982 vs. 76 fronds in the KCl plus 2,4-DNT and $CaCl_2$ plus 2,4-DNT systems, respectively. The results clearly show the exchange of Ca^{2+} for K^+ on the exchange sites of K-smectite in the $CaCl_2$ amended system induced the release of 2,4-DNT into solution. With the resultant increased bioavailability of 2,4-DNT, its toxic effects (manifested as lower frond numbers) were apparent by day 7 and thereafter.

Table 3. Comparison of duckweed frond numbers (average of four samples) at different times in K-smectite suspensions with 2,4-dinitrotoluene (2,4-DNT) and no 2,4-DNT present. At day 4, KCl or $CaCl_2$ solutions were added to the suspensions and the incubation was continued until 17 days. The standard errors of the means were all ≤ 1%. The ANOVA was applied to determine the statistical significance ($P<0.05$) for differences in treatments.

	Exposure time (days)						
Treatments	Day 04	Day 06	Day 07	Day 09	Day 11	Day 13	Day 17
2,4-DNT + KCl	19.5 ^{a}	41.3 ^{b}	54.1 ^{c}	106 ^{c}	200 ^{c}	407 ^{c}	982 ^{c}
2,4-DNT + $CaCl_2$	19.1 ^{a}	30.0 ^{a}	37.0 ^{a}	47.9 ^{a}	53.0 ^{a}	67.2 ^{a}	75.9 ^{a}
Control: no-DNT + KCl	20.1 ^{a}	41.7 ^{b}	56.3 ^{c}	104 ^{c}	209 ^{c}	395 ^{c}	1153 ^{c}
Control: no-DNT + $CaCl_2$	19.1 ^{a}	32.8 ^{a}	53.0 ^{b}	64.1 ^{b}	105 ^{b}	172 ^{b}	441 ^{b}

* Means followed by the same letter in the same column are not significantly different at the probability level ($\alpha = 0.05$)

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