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REDUCTIVE DEGRADATION OF *p,p′*-DDT BY Fe(II) IN NONTRONITE NAu-2

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Abstract—Clay minerals are abundant in soils and sediments and often contain Fe. Some varieties, such as nontronites, contain as much as 40 wt.% Fe_2O_3 within their molecular structure. Several studies have shown that various Fe-reducing micro-organisms can use ferric iron in Fe-bearing clay minerals as their terminal electron acceptor, thereby reducing it to ferrous iron. Laboratory experiments have also demonstrated that chemically or bacterially reduced clays can promote the reductive degradation of various organics, including chlorinated pesticides and nitroaromatics. Therefore, Fe-bearing clays may play a crucial role in the natural attenuation of various redox-sensitive contaminants in soils and sediments. Although the organochlorinated pesticide *p,p′*-DDT is one of the most abundant and recalcitrant sources of contamination in many parts of the world, the impact of reduced Fe-bearing clays on its degradation has never been documented. The purpose of the present study was to evaluate the extent of degradation of *p,p′*-DDT during the bacterial reduction of Fe(III) in an Fe-rich clay. Microcosm experiments were conducted under anaerobic conditions using nontronite (sample NAu-2) spiked with *p,p′*-DDT and the metal-reducing bacteria *Shewanella oneidensis* MR-1. Similar experiments were conducted using a sand sample to better ascertain the true impact of the clay *vs.* the bacteria on the degradation of DDT. Samples were analyzed for DDT and degradation products after 0, 3, and 6 weeks of incubation at 30ºC. Results revealed a progressive decrease in *p,p′*-DDT and increase in *p,p′*-DDD concentrations in the clay experiments compared to sand and abiotic controls, indicating that Fe-bearing clays may substantially contribute toward the reductive degradation of DDT in soils and sediments. These new findings further demonstrate the impact that clay materials can have on the natural attenuation of pollutants in natural and artificial systems and open new avenues for the passive treatment of contaminated land.

Key Words—DDT, Fe-bearing Clay, Fe-reducing Bacteria, Nontronite NAu-2, Organochlorinated Pesticide, Reductive Degradation, *Shewanella oneidensis* MR-1.

INTRODUCTION

Pesticides have been used throughout history, with evidence showing that the Sumerians used natural insecticides to protect against fleas, lice, and other pests ~4500 y ago (*e.g.* McKinney and Schoch, 2003). The types of active substances used have changed drastically over time. The research, development, and mass production of organic pesticides increased in the early 20th century, with the development of the synthetic chemical industry. At that time, an effective pesticide was seen to be one that had a strong and rapid toxicity to pests, a good chemical stability, a low production cost, and a relatively low toxicity to humans and non-target animals and plants (Mellanby, 1992). Organochlorinated compounds, such as *p,p′*-DDT (1,1,1-trichloro-2,2-bis (*p*-chlorophenyl)ethane or dichlorodiphenyltrichloro ethane; Figure 1), were among the first synthetic organic pesticides to be commercially produced and used around the world. From its first commercial production in 1943, *p,p′*-DDT became a very popular pesticide as it suited the ideals of the time – it was cheap to produce, very toxic to insects, highly stable, and was thought to have a low toxicity to humans (Kale *et al.*, 1999). The *p,p′*-DDT has been used for many years to protect agricultural crops and to prevent the spread of vector-borne diseases such as malaria and typhus (*e.g.* Turusov *et al.*, 2002; ATSDR, 2002). An estimated 2.8 billion kg of *p,p′*-DDT were produced and used around the world as a commercial or domestic pesticide between 1943 and 1974, where ~80% was used for agriculture (Stenersen, 2004; Turusov *et al.*, 2002). In the early 1960s, it became clear that DDT and its degradation products (Figure 1) were highly persistent in the environment and were bioaccumulating in the fat-rich tissues of birds, humans, and other animals, resulting in long-term adverse effects. Particularly affected were non-target organisms that relied on insects and worms; the problem arising when the latter had been in direct contact with the pesticides (*e.g.* Turusov *et al.*, 2002; ATSDR, 2002; Tanabe *et al.*, 1994; Kelce *et al.*, 1995; Longnecker *et al.*, 1997; Jaga and Dharmani, 2003). For instance, DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene or dichlorodiphenylethylene; one of the primary metabolites of DDT) has been shown to cause egg-shell thinning in several protected species of birds of prey, such as bald eagles, peregrine falcons, white-faced ibises, and osprey,

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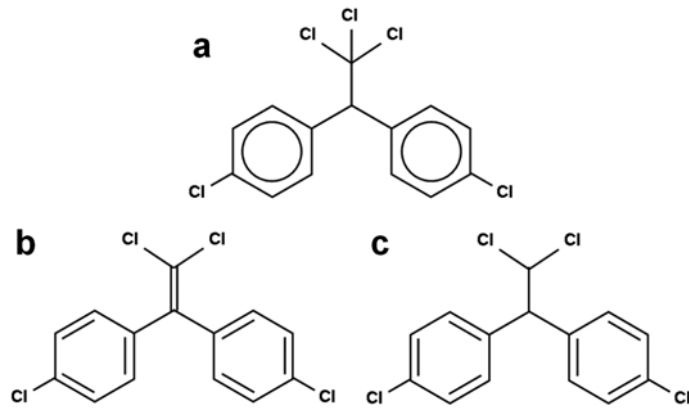

Figure 1. Molecular structure of *p,p′*-DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (a) and its primary metabolites, *p,p′*-DDE (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene) (b) and *p,p′*-DDD (1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) (c).

which considerably reduces the likelihood of survival of their chicks (Hurt *et al.*, 2003; Ewins *et al.*, 2003). DDT is also thought to cause detriment to human health (*e.g.* carcinogenic effects, premature births, tremors, and convulsions: Turusov *et al.*, 2002; ATSDR, 2002). Following these findings, the agricultural usage of DDT and other organochlorine pesticides was banned in most countries around the world in the 1970s and 1980s. The use of DDT for the control of mosquito-borne diseases such as malaria has also since been abandoned in many countries and has been largely replaced by the use of other less recalcitrant insecticides. Consequently, the worldwide usage of DDT has plummeted from ~40,000 tons per year in the 1970s to ~1,000 tons per year today. The debate about the ban of DDT continues, however, with some arguing that it puts wildlife ahead of people, leading to millions of potentially preventable deaths in malaria-infested regions around the world because alternative pesticides currently used to control mosquito-borne diseases are more expensive and less efficient than DDT (*e.g.* Edwards, 2004). The prolonged use of DDT has, however, clearly resulted in the accumulation of large amounts of DDT and degradation products in soils, sediments, and waters throughout the world and "no living organism [on the planet] may be considered DDT-free" (Turusov *et al.*, 2002).

Numerous studies have been conducted over the last two decades to better understand the mechanisms of transport and degradation of DDT and to develop cost-efficient remediation technologies.

Technical grade *p,p′*-DDT generally contains up to 15% of its ortho-isomer *o,p′*-DDT plus traces of its two primary metabolites DDD (1,1-dichloro-2,2-bis(*p*-chloro phenyl)ethane) and DDE (*e.g.* An *et al.*, 2005; ATSDR, 2002; Figure 1). Collectively, DDT and its metabolites are referred to as ΣDDT. They are all highly non-polar lipophilic compounds that have a low aqueous solubility ranging from 0.025 mg/L for *p,p′*-DDT to 0.14 mg/L for *o,p′*-DDE (ATSDR, 2002). They also have a very high affinity for organic matter, with an organic carbon partition coefficient (K_{oc}) ranging from $10^{4.70}$ for *p,p′*-DDE to $10^{5.35}$ for *o,p′*-DDT (ATSDR, 2002). Hence, ΣDDT is readily taken up by fatty tissues of animals and humans where it accumulates and, therefore, also tends to remain longer in soils and sediments with large organic-matter contents.

Depending on environmental conditions, the half life of DDT in soils and sediments is known to range from 2 to 15 y (*e.g.* Fiedler, 2003; ATSDR, 2002; Turusov *et al.*, 2002). Furthermore, compounds can become more strongly associated with soil components over time and become less bioavailable, a process known as 'aging' (Gevao *et al.*, 2000). Half lives for DDT and its metabolites are estimated to be more than twice as long in Australia where cold and wet winters and hot and dry summers increase the aging process. Hence, although DDT has been partially banned in agriculture for >20 y, DDT and its metabolites are still very much present in the environment throughout the world, with ΣDDT concentrations in soils often exceeding a few mg/kg (Aislabie *et al.*, 1997) and sometimes reaching concentrations as high as a few g/kg (US EPA, 2005). A slow but continuous decrease in ΣDDT concentrations is generally observed in contaminated soils and sediments, resulting from erosion, migration, plant and animal uptake, photodegradation, and biodegradation (Aislabie *et al.*, 1997).

Migration of ΣDDT is particularly frequent in sandy soils where the organic-matter content is small. Because the secondary metabolites (DDE and DDD) are more soluble than DDT, they are more mobile and hence are often transported *via* surface and groundwater flows into rivers and lake sediments. Because ΣDDT compounds are volatile, they also tend to migrate from temperate zones in low–mid latitudes to the high-latitude zones, such as the Arctic and Antarctic and persist for longer in these colder climatic regions (ATSDR, 2002).

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Photodegradation of *p,p′*-DDT to metabolites DDD and DDE has been demonstrated experimentally on leaf and soil surfaces by irradiation under UV light (Dolinova *et al.*, 2004; Quan *et al.*, 2005).

The biodegradation of *p,p′*-DDT due to various organisms (*i.e.* bacteria, fungi) is known to occur naturally in the environment by two main routes: oxidative degradation under aerobic conditions and reductive degradation under anaerobic conditions. Under aerobic conditions, *p,p′*-DDT is generally converted to *p,p′*-DDE, usually by dehydrochlorination by bacteria (Aislabie *et al.*, 1997). Aerobic degradation also tends to promote ring cleavage and occurs more quickly in warm and moist conditions (Fiedler *et al.*, 2003). Although few studies have shown that DDE can be further degraded under anaerobic and aerobic conditions (*e.g.* Quensen *et al.*, 1998; Hay and Focht, 1998), DDE is generally recalcitrant to microbial degradation and under most conditions is, therefore, seen as a 'dead-end' metabolite. DDE is the most widespread and abundant of all organochlorine pesticide residues found in British and other wildlife surveys (Mellanby, 1992).

In their review paper on the microbial degradation of DDT, Aislabie and co-authors referred to several studies that have observed enhanced *p,p′*-DDT degradation under reducing conditions when a carbon source is available (Aislabie *et al.*, 1997). The degradation process is due mainly to the actions of facultative anaerobic micro-organisms and also to chemical reactions in the soil. Anaerobic degradation generally converts *p,p′*-DDT to *p,p′*-DDD by reductive dechlorination (hydrogenolysis), where a single chlorine atom is substituted by a hydrogen atom. Although *p,p′*-DDD alone was a regulated pesticide, it can undergo further degradation to less harmful metabolites, such as 1-chloro-2,2-bis(*p*-chlorophenyl)ethylene (DDMU) and 1-chloro2,2-bis(*p*-chlorophenyl)ethane (DDMS) (Aislabie *et al.*, 1997).

Although the anaerobic degradation of DDT is generally slow, a number of laboratory studies has shown that DDT can be converted "rapidly" to DDD by certain soil micro-organisms under anaerobic conditions (Aislabie *et al.*, 1997). For example, Guenzi and Beard (1967) showed that the incubation of a soil sample spiked with DDT labeled with ^{14}C under anaerobic conditions resulted in the direct conversion of nearly 80% of DDT to DDD in only 4 weeks. Though no degradation was observed when sterilizing the soil sample before incubation, the results presented do not show whether the solid phases (possibly clay constituents) played any role in the 'rapid' biodegradation of DDT.

Clay minerals are widespread and often abundant in soils and sediments. Due to their small particle size, large surface area, and often large cationic exchange capacity (CEC), clay minerals play a key role in many biogeochemical processes. Many varieties of clay minerals, such as smectite, may contain substantial amounts of Fe(III) in their structure. In particular, nontronites may contain up to ~40 wt.% Fe_2O_3 as structural Fe(III) (*e.g.* Gates *et al.*, 2002). Numerous laboratory studies have demonstrated that the structural Fe(III) in smectite clay minerals, including nontronites, can be reduced under anaerobic conditions to Fe(II) by chemical reductants (*e.g.* dithionite, sulfide, and thiosulfate; Stucki *et al.*, 1996; Fialips *et al.*, 2002) or Fe-reducing bacteria (*e.g.* bacterial cultures from rice-paddy soils and other well drained and flooded soils; commercially available *Shewanella*, *Geobacter*, *Pseudomonas*, and *Bacillus* species; Kostka *et al.*, 1996, 1999a, 1999b; Stucki, 2006). Unlike Fe (oxyhydr)oxides which usually dissolve upon Fe reduction, minimal, if any, dissolution of structural Fe occurs in natural Fe-bearing smectites upon bacterial reduction, except in the presence of a chelating agent, such as nitrilotriacetic acid (NTA; Kostka *et al.*, 1999a; Stucki and Kostka, 2006). The bacterial reduction is also generally reversible (complete reoxidation to Fe(III); Stucki, 2006; Fialips *et al.*, 2002; Komadel *et al.*, 1995).

Many bench-scale studies have demonstrated that chemically or bacterially reduced Fe-bearing smectites can promote the reductive degradation of various organic compounds, such as pesticides (Xu *et al.*, 2001), chlorinated aliphatics (Cervini-Silva *et al.*, 2003), and nitroaromatics (Yan and Bailey, 2001). Pesticides studied include atrazine, dicamba, alachlor, oxamyl, 2,4-D, trifluralin, and chloropicrin (Stucki, 2006).

Though DDT is one of the most abundant and recalcitrant sources of contamination in many parts of the world, the impact of reduced Fe-bearing clays on its degradation has, however, never been documented. The main hypothesis of the present study was that bacterially reduced Fe-bearing clay minerals can substantially contribute to the reductive dechlorination of DDT to DDD according to the simplified mechanism presented (Figure 2). Fe-bearing clay minerals would, therefore, play a crucial role in the natural attenuation of DDT under anoxic conditions in soils and sediments and could be used in natural and artificial systems for the remediation of DDT contaminations.

The objective of this study was to test this hypothesis by studying the impact of bacterial reduction of an Fe-rich smectitic clay on the anaerobic degradation of *p,p′*-DDT.

MATERIALS AND METHODS

Mineral materials

The nontronite, NAu-2, from the Uley Mine, South Australia, was obtained from the Source Clays Repository of The Clay Minerals Society (Keeling *et al.*, 2000). NAu-2 is an Al-poor and Fe-rich clay containing ~38 wt.% Fe_2O_3 (Gates *et al.*, 2002). Mineralogical analyses of the bulk NAu-2 clay show the presence of ~95% nontronite (<2 μm), traces of

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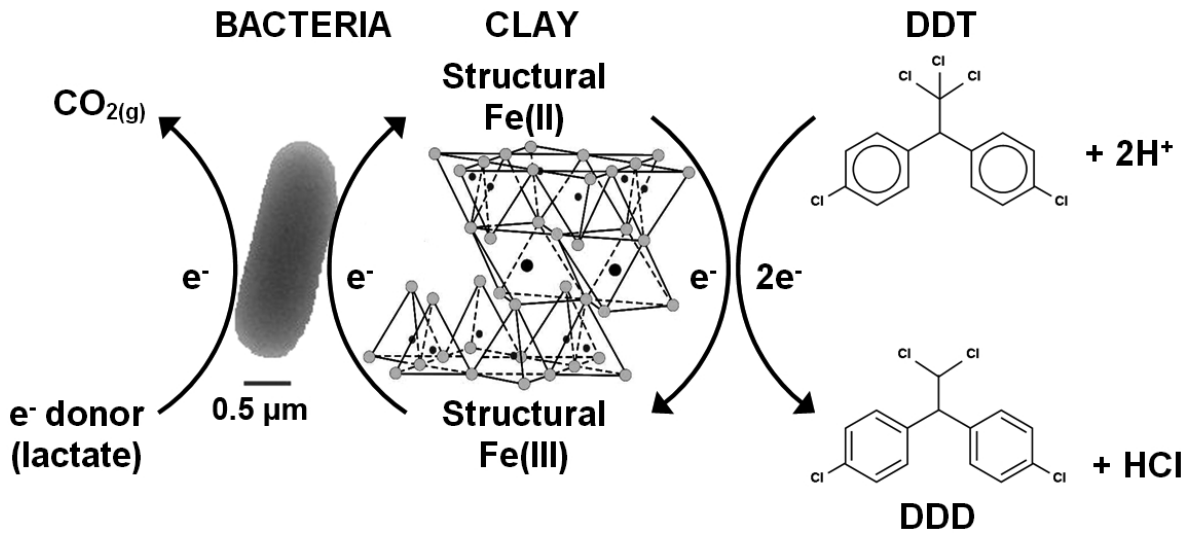

Figure 2. Simplified biogeochemical pathway for the reductive dechlorination of DDT to DDD by a reduced Fe-bearing clay.

quartz and Fe (oxyhydr)oxides (<1%), and <5% plagioclase feldspar (Keeling *et al.*, 2000).

The bulk clay was dried overnight at 105ºC and crushed for a few minutes using a Retsch RM100 crusher. The crushed clay was then passed through a 35-mesh sieve to obtain a fine and homogeneous powdery material of <0.5 mm. Chemical analyses of the raw sample indicated a total organic carbon content (TOC) of 0.16±0.03 wt.%. The overall Fe content of the material measured after complete acid digestion was 41.7 wt.% Fe_2O_3, with an Fe(III)/total Fe ratio of 99.08±0.01% (measured by UV-Visible spectroscopy with 1,10-phenanthroline using the method developed by Stucki and co-workers; *e.g.* Stucki, 1981; Komadel and Stucki, 1988).

A relatively pure silica sand (Levenseat LV60; 99.3% SiO_2), provided by a local supplier (WBB Minerals-Sibelco UK, Ltd.), was also used in this study to replace the nontronite clay in parallel experiments. The sand was sieved to obtain the 63 to 354 µm fraction. The TOC of the sand sample was 0.04±0.01 wt.% and the Fe content was only 0.06±0.03 wt.% Fe_2O_3 (measured by X-ray fluorescence), with an Fe(II)/total Fe ratio of 14.6±0.1% (measured by UV-Visible spectroscopy using the 1,10-phenanthroline method; the given error represents the propagated error for triplicate analyses on the same digested solution).

Solvents, organic standards, and other chemicals

All solvents, organic standards, and other laboratory chemicals used in this study were purchased from Sigma Aldrich, Ltd. (Gillingham, Dorset, UK), Fisher Scientific, Ltd. (Loughborough, UK), or VWR International, Ltd. (Lutterworth, UK). The solvents (methanol, acetone, dichloromethane, and hexane) were pesticide-grade reagents, ⩾99% pure. The organic standards were all certified ⩾98.0% or ⩾99.0% pure. Florisil (magnesium silicate) with a grain size between 74 and 149 µm (100–200 mesh), analytical grade sodium sulfate, and 1,10-phenanthroline were ⩾99.0% pure. All the chemicals required for the preparation of the minimal culture medium were obtained as Puriss (⩾99.0%) or Biochemica Ultra (⩾99.5%) solids.

Hexane was used as solvent for preparing the following organic standard solutions: 196.0 µg/mL *p,p′*-DDT, 207.9 µg/mL *o,p′*-DDT, 232.8 µg/mL *p,p′*-DDD, 166.4 µg/mL *o,p′*-DDD, 169.6 µg/mL *p,p′*-DDE, 166.4 µg/mL *o,p′*-DDE, 200.0 µg/mL *p,p′*-DDMU, 196.98 µg/mL Lindane, and 104.6 µg/mL octafluoronaphthalene (OFN). A standards calibration mix (ΣDDT/Lindane standard mix) was prepared by mixing known volumes of the standard solutions to obtain the following composition: 19.60 µg/mL *p,p′*-DDT, 20.79 µg/mL *o,p′*-DDT, 23.28 µg/mL *p,p′*-DDD, 16.64 µg/mL *o,p′*-DDD, 16.96 µg/mL *p,p′*-DDE, 16.64 µg/mL *o,p′*-DDE, 20.00 µg/mL *p,p′*-DDMU, and 19.70 µg/mL Lindane. The organic standard solutions and the ΣDDT/Lindane standards mix were transferred to air-tight glass vials and stored at 4ºC until use. More concentrated solutions of *p,p′*-DDT (2451.0 µg/mL) and Lindane (1923.7 to 2533.3 µg/mL) were also prepared with hexane and acetone to be used as standards in Soxhlet extractions.

Fe-reducing bacteria

Shewanella bacteria are among the most studied Fe(III) reducers (Lovley, 1997). They can use a variety of electron acceptors, including Fe(III) within the structure of Fe-bearing minerals (*i.e.* oxides, oxyhydroxides, and clay minerals). Being facultative anaerobes, they are convenient for use in both laboratory and field

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situations as they can be quickly cultured under aerobic conditions before acclimation under anaerobic conditions for subsequent Fe-reducing applications. They grow naturally almost anywhere, making them ideal for use in bioremediation studies.

Shewanella oneidensis (*S.o.*) strain MR-1 was obtained from NCIMB, Ltd. (Aberdeen, UK) as a pure lyophilized culture. MR-1 was isolated originally from anoxic sediments of Lake Oneida, New York (Myers and Nealson, 1988; Venkateswaran *et al.*, 1999) and has been studied widely and used in many laboratory studies of bacterial Fe-reduction in clay minerals and oxides (*e.g.* Kostka *et al.*, 1999a).

A standard approach using Tryptic Soya Broth (TSB) was used to revive the lyophilized cells of *S.o.* MR-1 and to culture them under aerobic conditions, as recommended by NCIMB, Ltd. The TSB solution was prepared by dissolving TSB powder (7.5 g) in deionized water (500 mL) and sterilized by autoclaving at 121ºC for 20 min. A dilution series of the lyophilized cells in TSB (0, 5, 25, 125, and 625X) was used to create five cultures with a range of population densities and, therefore, identify the optimum growth conditions for subsequent sub-cultures.

All vials were left in a 30ºC incubator overnight on an orbital shaker (320 rpm). Additional open and closed vials with no bacteria added were set up as controls. No microbial contamination was ever observed in any of the control vials. To prevent nutrient availability becoming a limiting factor, the bacteria were cultured using the optimum dilution (5 × dilution); when the cultures were estimated to be at mid-log growth phase, they were sub-cultured once into a fresh medium and incubated at 30ºC for 24 h with continuous shaking (320 rpm).

Cell counting

The SYBR gold nucleic acid stain was used to assess the number of bacteria present in the culture stock before use in the subsequent experiments. 500 mL of cell suspension from the subculture and the same from the open and closed controls was placed in 1 mL Eppendorf tubes. 500 µL of sterile 50% ethanol was then added. These 1 mL samples were then stored at −20ºC prior to cell counting. To perform cell counts, an aliquot (100 µL) of the ethanol fixed sample was added to a filter sterile phosphate buffered saline solution (1 × PBS 850 µL). Then 50 µL of 100 × diluted SYBR gold nucleic acid stain in PBS was added. The samples were wrapped in Al foil (as SYBR gold is light sensitive) and incubated at room temperature for 30 min. They were then vacuum filtered through a 0.2 µm pore-size Isopore membrane and washed three times with 1 mL of filter-sterilized water. A drop of Citifluor (glycerol in PBS) was placed on a clean microscope slide to act as a 'glue' and the filter transferred onto the microscope aseptically. Another drop of Citifluor was added to the filter and a cover slip placed on top. The slides were stored in the dark to prevent the fluorescence from fading. An Olympus BX40 Epi-fluorescence microscope and Olympus C-2020 Z camera were used to take photographs of the slides using 100 × oil immersion under a blue light filter in the dark. Twenty photographs were taken at random for each slide, ten focused through the microscope lens and ten through the camera screen. *Cell-C* image-analysis software (Selinummi *et al.*, 2005) was used to count the cells in each photograph. The mean initial count for the sub-culture stock was $1.59 \pm 0.08 \times 10^9$ cells mL^{-1}.

Microcosm experiments

Most studies focusing on the bacterial reduction of Fe in clay minerals use clay suspensions with low clay loading (generally ~4−5 g/L; Kostka *et al*., 2002; Jaisi *et al*., 2005). To better simulate conditions of a contaminated soil, the present study was conducted with a much greater mineral loading (196 g/L). Precisely weighed amounts of clay or sand (10 g) were placed in 100 mL borosilicate glass serum bottles. The bottles and their rubber septa were then sterilized in an autoclave at 121ºC for 20 min and immediately placed in a biological safety cabinet.

A solution of 2305.94 µg/mL *p,p′*-DDT was prepared by adding 235.3 mg of DDT (98% purity) to 100 mL acetone. One mL of the DDT solution was transferred into each serum bottle, directly on the clay or sand samples, using a sterile 1 mL pipette. The bottles were then left open overnight in the biological safety cabinet to allow the acetone to evaporate with minimal opportunity for microbial contamination to occur.

Fifty mL of a minimal culture medium, adapted from Nealson and Scott (2006) and Kostka *et al.* (1999a, 199b), was then added, with the following composition: 9.01 mM $(NH_4)_2SO_4$, 5.69 mM K_2HPO_4, 0.002 mM $NaHCO_3$, 3.28 mM KH_2HPO_4, 1.01 mM $MgSO_4$, 0.48 mM $CaCl_2$, 0.067 mM Na_2EDTA, 0.012 mM NaCl, 0.006 mM $CoSO_4$, 0.006 mM $Ni(NH_4)_2(SO_4)_2$, 20 mg/L L-arginine, 20 mg/L L-glutamate, 20 mg/L L-serin, and 30.00 mM lactate (carbon source). For experiments with the nontronite, structural Fe(III) was the only electron acceptor. Because the sand sample contained no significant amounts of electron acceptor (very small Fe content), a suitable amount of an electron acceptor (ferric citrate) had to be added to allow bacterial growth. Tests in the laboratory have shown that, even though the surface properties (grain size, surface area, and reactivity) of the sand are very different from those of the nontronite NAu-2, a similar bacterial growth rate of *S.o.* MR-1 can be achieved with the sand compared to that achieved with the nontronite clay by adding 50.0 mM of ferric citrate. Hence, parallel microcosm experiments with the sand were run with a different minimal culture medium, containing 50 mM ferric citrate, to evaluate the true impact of the reduced clay *vs.* the bacteria on DDT degradation.

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Each serum bottle was then flushed with O_2-free N_2 gas for a minimum of 8 min through a sterile 2 μm filter and needle and sealed. One mL of the bacteria sub-culture stock obtained after 24 h of incubation in TSB ($1.59\pm0.08 \times 10^9$ cells/mL) was aseptically transferred to a 50 mL sterile centrifuge vial and centrifuged for 10 min at 4000 rpm. The supernatant, consisting of the TSB growth media, was poured off and the bacterial pellet was re-suspended in 25 mL of the fresh experimental culture medium (Fe free). After centrifugation and removal of the supernatant, the bacterial cells were re-suspended a second time in 25 mL of fresh culture medium. One mL of the bacterial culture was then immediately injected into the serum bottles and the headspace of each microcosm was degassed for a further 2 min with O_2-free N_2 gas to quickly establish anaerobic conditions. The initial cell density was expected to be $0.12\pm0.01 \times 10^7$ cells/mL (dilution factor of 1275).

The sealed and crimped serum bottles were then either stored at −80ºC immediately after degassing of the headspace (considered time-zero) or placed upside down on a shaking incubator (320 rpm) at 30ºC for 3 or 6 weeks. All experiments were carried out in triplicate.

A further set of experiments was conducted in parallel under the same conditions for 0, 3, and 6 weeks of incubation, exclusively for the analyses of cell density, Fe reduction, and headspace gases. As the analyses of bacterial density and Fe speciation are destructive, these samples could not be analyzed for *p,p′*-DDT and its degradation products. Three control experiments were also conducted for the nontronite with no bacteria added for 0, 3, and 6 weeks of incubation.

Extraction of DDT and degradation products

Soxhlet extractions. The samples (sacrificed serum bottles, in triplicate, after 0, 3, and 6 weeks of incubation) were frozen at −80ºC overnight and freeze dried at −15ºC. Once dry, each solid sample was transferred to a cellulose extraction thimble and placed in a Soxhlet extractor. Anhydrous sodium sulfate (~10 g) was added to each sample to ensure complete removal of any remaining water and a similar amount was also used in a separate Soxhlet extraction procedure to provide a procedural blank. All samples were spiked with 1 mL of a freshly prepared Lindane solution (1923.7−2297.8 μg/mL) as an extraction (surrogate) standard. The samples and blank were extracted for 18 h with 200 mL of dichloromethane:methanol (93:7) solvent. After cooling, the contents of each round-bottom flask were rotary evaporated to near-dryness in a 36ºC water bath, rinsed into 10 mL vials, and made up to 10 mL using dichloromethane (DCM) solvent.

Florisil columns. A Florisil column procedure modified from a US EPA (2007a) method was used for cleaning up the organic extracts. The Florisil was activated by heating at 650ºC for 4 h on a porcelain dish in a muffle furnace before being allowed to cool and transferred to an amber glass jar for storage. Glass chromatography columns (70 mm long, 13 mm internal diameter) were prepared by packing with activated Florisil (3 g) and anhydrous sodium sulfate (10 mm bed height). Each column was pre-eluted with hexane (40 mL, which was then discarded). An aliquot (200 μL) of the 10 mL sample extract solutions was added to the top of the columns and the columns were eluted with 4:1 hexane:DCM (30 mL). The eluates collected were evaporated to near dryness using a rotary evaporator and transferred to vials in hexane (1 mL). An aliquot (200 μL) of each eluate was transferred into an autosampler vial and made up to 1 mL using hexane. A 100 μL aliquot of a freshly prepared OFN solution (88.9 to 95.5 μg/mL in hexane) was added to each sample as an internal standard.

Analytical methods

Gas chromatography-mass spectrometry analyses (GC-MS). The eluate aliquots were analyzed by GC-MS using a Hewlett-Packard 6890 GC with a split/splitless injector (injector port temperature 280ºC) linked to a Hewlett-Packard 5973MSD quadrupole mass spectrometer (electron voltage 70 eV, filament current 220 μA, multiplier voltage 2600 V). The GC-MS was fitted with a 30 m silica capillary column (Agilent HP-5; 0.25 mm internal diameter, coated with a 0.25 μm film of 5% phenylmethylpolysiloxane). Aliquots (1 μL) of the fractions were injected in splitless mode by an HP7683 autosampler and the split opened (30 mL/min) after 1 min. After the solvent peak had passed, the temperature program and data acquisition commenced. The GC was temperature programmed to increase from 40 to 300ºC at 10ºC/min and held at the final temperature for 5 min. The carrier gas was He, with a flow rate of 1 mL/min (initial pressure of 50 kPa). The quadrupole temperature was 150ºC and the ion source temperature was 230ºC. The data were acquired in full scan mode at 50−400 a.m.u./s using *ChemStation* software.

A total of nine compounds was analyzed: *p,p′*-DDT, *o,p′*-DDT, *p,p′*-DDD, *o,p′*-DDD, *p,p′*-DDE, *o,p′*-DDE, *p,p′*-DDMU, Lindane, and OFN (internal standard). Quantification was achieved using a five-point calibration curve ranging from 0 to 10 μg/mL obtained by dilution of 5 to 500 μL of the ΣDDT/Lindane standard mix and 100 μL of 104.6 μg/mL OFN in a 1100 μL volume. As recommended by the US EPA (2007b), individual relative response factors (RRF; response factor of the analyte normalized to the response factor of the internal standard) were determined for each analyte at each calibration level and mean RRF values were calculated (Table 1; Appendix). The percent recovery was also determined to evaluate the efficiency of the extraction using the surrogate standards (Appendix).

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Table 1. Retention time, mean relative response factor (Mean RRF), relative standard deviation (%RSD), and correlation coefficients (R^2) for each analyte.

Analyte	Retention time (min)	Mean RRF	%RSD	R^2
p,p′-DDT	20.596	0.591	4.38	0.9998
o,p′-DDT	19.978	0.773	4.70	0.9999
p,p′-DDE	19.142	0.561	3.88	0.9996
o,p′-DDE	18.557	0.910	8.48	0.9998
p,p′-DDD	19.932	1.237	0.73	1.0000
o,p′-DDD	19.311	1.118	6.86	0.9997
p,p′-DDMU	18.480	0.629	1.67	1.0000
Lindane	15.062	0.444	5.77	0.9997

Analysis of headspace gases. To ascertain that the microcosms were under anaerobic conditions, GC-MS analysis of headspace gases was performed after 0, 3, and 6 weeks of incubation on the two separate sets of experiments (not analyzed for ΣDDT) with the nontronite and sand substrates.

The analyses were carried out on a Fisons 8060 GC using split injection (250ºC) linked to a Fisons Trio1000 MS (electron voltage – 70 eV, filament current – 4.2 A, source current – 1000 μA, source temperature – 250ºC, multiplier voltage – 500 V, interface temperature – 250ºC). Data acquisition (using MassLab software) was in full-scan mode (0.5–100.5 a.m.u.) or in SIM mode of ten ions for greater sensitivity. The headspace sample (100 μL) was taken with a He flushed gas-tight syringe (SGE, Australia) and injected with the split open. Separation was performed on a HP-PLOT-Q capillary column (30 m × 0.32 mm i.d.) packed with 20 μm Q phase. The GC oven temperature was held isothermally at 35ºC and He was used as the carrier gas (flow 1 mL/min, inlet pressure 65 kPa, split at 100 mL/min). N_2, O_2, and CO_2 were detected by single-ion monitoring (m/z = 28, 32, and 44, respectively).

Analysis of the Fe-reduction level in the sand and clay samples. The Fe(II)/total Fe ratio of the raw sand and nontronite samples and that of the nontronite samples after 3 and 6 weeks of incubation (separate set of experiments, not analyzed for ΣDDT) were measured by UV-Visible spectroscopy using the 1,10-phenanthroline (phen) method developed by Stucki and co-workers (*e.g.* Stucki, 1981; Komadel and Stucki, 1988). Briefly, the Fe(II) concentration was first measured by photochemical analysis of the $[Fe(phen)_3]^{2+}$ complex formed during HF-H_2SO_4 digestion of ~20 mg of the material under red light in the presence of an excess of 1,10-phenanthroline. Then the total Fe concentration was measured after converting any complexed Fe(III) in the digestate to $[Fe(phen)_3]^{2+}$ by chemical reduction using hydroxylamine hydrochloride (NH_3OHCl).

Recovery experiments

A number of trials was carried out in order to quantify the validity of each stage of the extraction and analytical procedures. In particular, *p,p′*-DDT is known to break down at high temperatures such as those found in the GC injector and column (Gfrerer and Lankmayr, 2005). The extent of this breakdown was quantified several times throughout the study.

p,p′-DDT is also known to adsorb to organic matter (WHO, 1989; ATSDR, 2002). Such adsorption may affect the DDT extraction yield from solids containing organic matter. Because the clay and sand samples contain small proportions of organic matter, the extraction recovery of *p,p′*-DDT from these two mineral materials was tested. Degradation products of DDT (DDE and DDD) are also known to adsorb to organic matter but to a similar or smaller extent (K_{oc} ranging from $10^{4.70}$ for *p,p′*-DDE to $10^{5.19}$ for *o,p′*-DDD compared to $10^{5.18}$ for *p,p′*-DDT and $10^{5.35}$ for *o,p′*-DDT; ATSDR, 2002); their extraction recoveries from the clay and sand materials were not tested.

Breakdown of the DDT standard in the 30 m column. A 5 μg/mL *p,p′*-DDT standard was prepared and analyzed several times using GC-MS to check the cleanliness of the injection port and quantify DDT breakdown in the 30 m column. Further *p,p′*-DDT standard runs were also completed throughout the study before and after every 6–8 samples.

The percentage of DDT breakdown was calculated using equation 1.

$$\%\text{Breakdown}_{\text{DDT}} = \frac{100 \times \Sigma(A_j/(\text{MeanRRF}_j \times M_j))}{\Sigma(A_i/(\text{MeanRRF}_i \times M_i))} \quad (1)$$

with the A_j, MeanRRF_j, and M_j terms corresponding to the peak area, mean RRF value (Table 1), and molecular weight of the breakdown product j (DDE and DDD), respectively. The terms A_i, MeanRRF_i, and M_i correspond to the peak area, mean RRF value, and molecular weight of all the analytes i (DDT, DDE, and DDD), respectively.

According to the US EPA (2007b), an acceptable range of DDT degradation in the injector port is 15% or less.

Soxhlet extraction efficiency. Soxhlet extractions were carried out to verify that recoveries from the clay and sand were acceptable. Aliquots (10 g) of the clay and sand were spiked with 1 mL of *p,p′*-DDT solution (2451.0 μg/mL) and 1 mL of Lindane solution (2533.3 μg/mL). The two extractions were conducted for 18 h using 250 mL of a DCM:methanol (93:7) mixture and the extracted solutions were reduced by rotary evaporation before being put through the Florisil column. The eluted solvents were then rotary evaporated

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further and then diluted to a known volume for GC-MS analysis.

Florisil column efficiency. The Florisil column efficiency was assessed by adding 200 µL of the standard *p,p′*-DDT solution to the top of the column and eluting with 30 mL of 4:1 hexane:DCM. The collected samples were rotary evaporated and brought to a final expected concentration of ~3.92 µg/mL with hexane. The test was conducted in triplicate.

A 3.92 µg/mL *p,p′*-DDT solution was also prepared by diluting 200 µL of the standard solution in 800 µL of DCM to allow comparison with the eluted solutions.

A 200 µL aliquot of each sample (eluted and non-eluted) and 800 µL of hexane were transferred into GC vials and an aliquot (100 µL of 104.6 µg/mL) of OFN internal standard solution was added to each sample.

RESULTS AND DISCUSSION

Recovery experiments

Breakdown of the DDT standard in the 30 m GCMS column. Seven analyses of the *p,p′*-DDT standard were completed at the beginning of the study (Table 2) and all DDT recoveries were within the acceptable range given by the US EPA (2007b), *i.e.* 80−110%, with an average %Breakdown of only 2.8±0.5% (%Recovery = 97.2±0.5%). The only other organic species detected was *p,p′*-DDD. Because the *p,p′*-DDT standard used in this study was only 98.0% pure, it may well have contained up to 2% *p,p′*-DDD as an impurity. Hence, only 1 or 2% of the injected *p,p′*-DDT may have actually degraded in the column.

Other analyses of the *p,p′*-DDT standard completed before and after every 6−8 samples were also all within the acceptable range given by the US EPA. No organic compound other than those injected or which might be formed through breakdown of the injected compounds was observed in any of the chromatograms.

Soxhlet extraction efficiency. The percentages of DDT recovery obtained for the soxhlet efficiency trials using the two mineral samples were again well within the 80−110% guideline value (US EPA, 2007b; Table 3), with both mineral materials showing <4% loss. This value is very similar to the measured average %Breakdown of the *p,p′*-DDT standard in the GC-MS column (2.8±0.5%), suggesting that the soxhlet extraction is very effective, with little or no loss of DDT *via* irreversible adsorption to the mineral substrates or associated organic matter.

Table 2. Percentage of breakdown of the 5 µg/mL *p,p′*-DDT standard in the 30 m GCMS column.

Trial number	%Breakdown$_{DDT}$
1	3.66
2	2.93
3	2.97
4	2.62
5	2.56
6	2.15
7	3.00
Average	2.84
SD	0.47

Table 3. Results of soxhlet extraction of a *p,p′*-DDT solution from the two different mineral materials (*i.e.* the nontronite clay and the silica sand).

Mineral sample	%Recovery$_{DDT}$
Clay	96.43
Clay	96.81
Clay	96.51
Average	96.58
SD	0.20
Sand	96.61
Sand	98.15
Sand*	–
Average	97.38
SD	1.09

* One of the triplicate runs for the soxhlet extraction from the sand failed due to the presence of an organic impurity in the GCMS injection port or column.

Florisil column efficiency. The mean %Recovery of the *p,p′*-DDT standard after Florisil clean-up (92.1±0.9%) was slightly less than the mean non-eluted recovery (95.2±0.9%; Table 4). Both values were, however, well within the acceptable range (US EPA, 2007b). In addition, results from the soxhlet extraction trials for the clay and sand, for which extracted samples were run through the Florisil column, gave even greater %Recovery (>96%; Table 3 values), suggesting that

Table 4. Results of recovery of the *p,p′*-DDT standard after Florisil column treatment.

Trial	%Recovery$_{DDT}$
Eluted 1	92.37
Eluted 2	91.09
Eluted 3	92.84
Average	92.10
SD	0.91
Non-eluted 1	94.38
Non-eluted 2	95.11
Non-eluted 3	96.24
Average	95.24
SD	0.94

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the mean %Recovery from the Florisil column (92.1%) was probably underestimated.

Headspace gases, bacterial growth, and Fe reduction (separate sets of DDT degradation microcosms, not analyzed for ΣDDT)

Headspace gases. All headspace gas analyses showed the presence of $CO_{2(g)}$ and $N_{2(g)}$. The presence of $N_{2(g)}$ was expected because the microcosms were purged with O_2-free $N_{2(g)}$ to establish anaerobic conditions at the beginning of the experiments. $CO_{2(g)}$ was also expected, as it is produced during micro-organism metabolism (Figure 2). At time 0, only a very small amount of microbially produced $CO_{2(g)}$ may be present. However, the minimal culture medium contains some $NaHCO_3$, which is expected to equilibrate with the headspace $CO_{2(g)}$. Trace amounts of $O_{2(g)}$ were also observed at time 0 for both the clay and sand experiments, indicating that the procedure of flushing with O_2-free $N_{2(g)}$ may not have been long enough to establish complete anaerobic conditions. However, extremely small amounts were observed (non quantifiable) and none of the headspace samples collected after 3 and 6 weeks of incubation showed any trace of $O_{2(g)}$, indicating that anaerobic conditions were reached and maintained throughout the duration of the experiments.

Bacterial growth. Results of cell-density measurements showed that, for both the sand and clay experiments, the cell density increased progressively over the course of the experiments (Figure 3). Cell growth was particularly rapid during the first week of incubation, with cell density increasing from $\sim 1 \times 10^6$ cells/mL to $>3 \times 10^7$ cells/mL. These results show that at the concentrations used, DDT was not toxic to the bacterial cells and that they were able to use the structural Fe of the nontronite and the ferric citrate added to the sand as electron acceptors. Though the clay experiments had significantly larger concentrations of Fe(III) (52.2 mmol in total) than the sand experiments (2.6 mmol), the cell growth rates in the clay and sand microcosm experiments were very similar, even slightly greater in the sand experiments. Fe in the clay experiments was exclusively within the clay structure, making it less accessible to the bacteria than the aqueous ferric citrate added to the sand samples (*e.g.* Lovley and Phillips, 1988; Tobler *et al.*, 2007; Tobler, 2007), possibly explaining why similar growth rates were successfully achieved with such a large difference in Fe content. Epi-fluorescence microscopy does not, however, allow distinction between the proportion of viable *vs.* dead cells in the cell suspensions tested. The measured cell densities only give an indication of the total population, including active and dead cells.

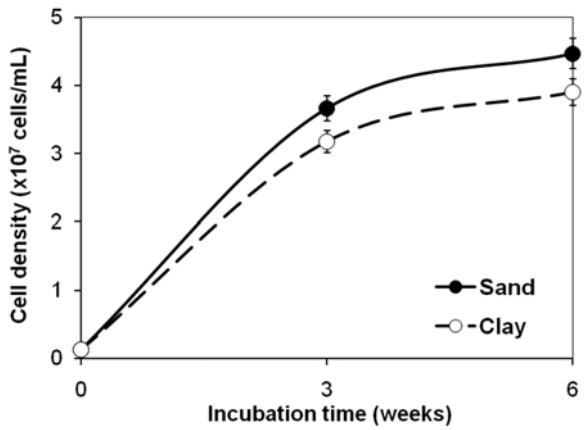

Figure 3. Cell growth in separate DDT-degradation experiments with the silica sand and nontronite clay. After injection of the concentrated cell solution in the serum bottles, the initial cell concentration was 0.12×10^7 cells/mL.

No cell growth was observed in any of the control vials, indicating that no microbial contamination was likely to have occurred during the preparation of the microcosm experiments.

Reduction level in the clay samples. Within 5 days of incubation with *S.o.* MR-1 at 30ºC, all the clay samples turned from yellow to green in color. Although the cell density in the clay and sand microcosm experiments were very similar, none of the sand samples (incubated for 0, 3, and 6 weeks) showed any change in color, indicating that the observed green coloration of the clay samples was not coming from the *S.o.* MR-1 cells. This green coloration rather suggests that Fe reduction occurred in the structure of the nontronite (*e.g.* Komadel *et al.*, 1990). The coloration changed from green after 3 weeks of incubation to dark green after 6 weeks of incubation, suggesting that the reduction level of the samples incubated for 6 weeks was substantially greater than that after 3 weeks of incubation.

Triplicate UV-Visible analyses of the nontronite clay for its reduction level after 3 and 6 weeks of incubation gave a fairly low Fe(II)/total Fe ratio, 1.03±0.01% after 3 weeks of incubation and 2.84±0.01% after 6 weeks. Repeated analyses after washing the samples using O_2-free deionized water gave similar results, indicating that the presence of DDT and its degradation products did not affect the UV-Visible analyses. The amount of clay used in the microcosm experiments being very large (10 g for 51 mL of solution), these small levels do correspond to large pools of Fe(II) (0.54 and 1.48 mmol of Fe(II), respectively), for a total number of cells of 1.6 and 2.0×10^9 cells, respectively.

The green coloration of the reduced clay does, however, indicate that the measured Fe(II)/total Fe ratio (1.0 and 2.8%) may be underestimated. A recent study conducted by White *et al.* (2008) found, for example, that, in the absence of an electron shuttle (AQDS: 9,10-anthraquinone-2,6-disulfonic acid), the reduction of the nontronite NAu-2 by a similar bacteria,

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S. algae BrY, leads to a very similar green coloration of the clay with a maximum reduction level of ~23% (Fe(II)/total Fe). The clay loading was, however, much smaller (2 g/L). Similarly, a study by Kostka *et al.* (2002) found a linear correlation between the number of cells of *S. oneidensis* MR-1 and the concentration of reduced structural Fe in the Fe-bearing smectite SWa-1. In their study, the percentage of structural Fe(III) reduced in the smectite SWa-1 remained at ~20% throughout the range of particle loading tested (0.5 to 4 g/L). Applying the linear relationship from Kostka *et al.* (2002), however, suggests that an absolute production of only ~0.043 mmol Fe(II) is expected for 2.0×10^9 cells of *S. oneidensis* MR-1, which is much smaller than in the present study (1.48 mmol Fe(II) for 2.0×10^9 cells). Other studies by Jaisi *et al.* (2005, 2008) have shown that the bacterial reduction of the nontronite NAu-2 for up to 200 days by *S.o.* CN32 cells in the absence or presence of AQDS leads to maximum reduction levels of ~14–18% and ~28–32%, respectively. The maximum reduction level is reached within 14 days with AQDS while reduction without AQDS is still ongoing after 200 days. The cell density reached by Jaisi *et al.* (2005) after 3 weeks of incubation in the absence of AQDS (~5×10^7 cells/mL) was relatively stable and in the same order of magnitude as the present study (~3.9×10^7 cells/mL), with a reduction level of the NAu-2 nontronite of ~10–14% after ~3 weeks of incubation and ~14–18% after ~6 weeks. Considering the reported clay loading of 5 mg/mL, the maximum concentration of Fe(II) obtained by these authors in 6 months of incubation (3.8 mM) corresponds, however, to a relatively small absolute amount of Fe(II) (0.076 mmol Fe(II) produced for a total amount of cells of ~10^9 cells), much smaller than that observed in the present study. If the same clay loading had been used in the present study, the maximum reduction levels of the nontronite NAu-2 after 3 and 6 weeks of incubation with *S.o.* MR-1 at 30ºC would be expected to range between 10 and 18%, with a significantly greater reduction level after 6 weeks of incubation than after 3 weeks. However, even though the electron donor was provided in excess (1.53 mmol of lactate, corresponding to a maximum of 18.36 mmol of electrons) and was, therefore, unlikely to be a limiting factor for bacterial growth and Fe reduction, the high clay loading used in the present study (196 mg/mL) may have substantially limited the availability of the structural Fe(III) to bacteria and the rate and extent of bacterial growth. In addition, some reoxidation is expected to occur upon the reductive dechlorination of DDT to DDD (Figure 2). A recent study by Day (2010) revealed, for example, extensive reoxidation of a reduced Fe-bearing smectite after its reaction with nitrate while nitrogen speciation clearly revealed substantial nitrate reduction to nitrite and ammonium. The reduction levels of the NAu-2 clays in the current study may be substantially smaller than 10–18% due to the clay loading and progressive reoxidation of the bacterially reduced clay through the reductive dechlorination of DDT to DDD as bacterial activity may not be sufficient or quick enough to maintain a large and constant (or increasing) level of reduction of Fe in the clay.

DDT-degradation experiments

Apart from the concentrations of *p,p′*-DDE and *p,p′*-DDD in the sand experiments (Table 5), all relative standard deviations (%RSD) obtained for the different analytes in the triplicate clay and sand microcosm experiments after 0, 3, and 6 weeks of incubation were small (<15%), indicating a satisfactory level of reproducibility (US EPA, 2007b). In addition, the corresponding %Recovery for the extraction standard (Lindane), total DDT compounds (DDT + DDE + DDD), and *p,p′*-DDT had excellent %RSD values, <8% (Table 6).

The recovery values of the extraction standard (Lindane; $\%Recovery_{Lindane}$) for the clay and sand microcosm experiments, the abiotic control experiments (clay with no bacteria added), and the blank recovery test (separate Soxhlet extraction with anhydrous sodium sulfate) were between 92.3 and 100.0%, which is well within the acceptable range (US EPA, 2007b). The total recovery of DDT compounds (ΣDDT; $\%Recovery_{\Sigma DDT}$) was also very large, ranging from 90.2 to 99.0%, indicating that the experimental methods were satisfactory, all possible degradation products were probably accounted for, and minimal DDT degradation occurred in the GC column.

A substantial and progressive decrease in the concentration of *p,p′*-DDT was observed with incubation time in the clay microcosm experiments (up to 22% compared to the initial *p,p′*-DDT concentration; Table 5; Figure 4), with a total absolute removal of 1.41 μmol of *p,p′*-DDT in 6 weeks. A progressive decrease in the

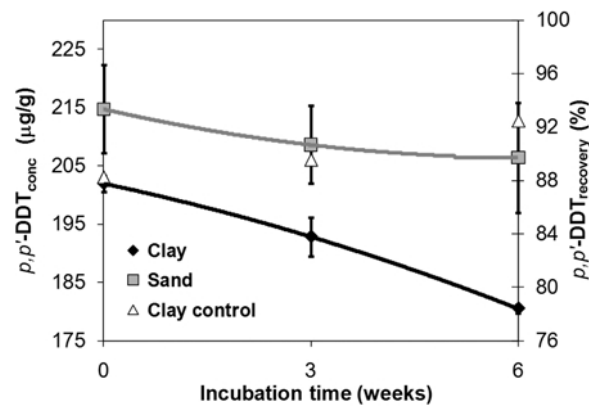

Figure 4. Results from triplicate experiments showing the measured concentration and %Recovery of *p,p′*-DDT for the nontronite clay and silica sand experiments after 0, 3, and 6 weeks of incubation. Concentrations measured in the clay controls (no bacteria added) are presented for comparison.

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Table 5. Mean concentration (μg/g), standard deviation (SD), and relative standard deviation (%RSD) of the different analytes for the triplicate clay and sand microcosm experiments after 0, 3, and 6 weeks of incubation. Results obtained for the control and blank experiments are also shown for comparison.

Mineral substrate	Incubation time (weeks)	Lindane in μg/g (SD) %RSD	*p,p′*-DDE in μg/g (SD) %RSD	*p,p′*-DDD in μg/g (SD) %RSD	*p,p′*-DDT in μg/g (SD) %RSD
Clay	0	219.63 (3.29) 1.50%	13.66 (1.67) 12.25%	9.72 (0.67) 6.91%	201.93 (1.34) 0.66%
Clay	3	203.93 (13.26) 6.50%	13.72 (1.47) 10.75%	11.96 (0.62) 5.21%	192.81 (3.32) 1.72%
Clay	6	187.03 (6.88) 3.68%	14.69 (0.62) 4.19%	14.33 (0.55) 3.82%	180.48 (0.77) 0.43%
Sand	0	211.96 (12.42) 5.86%	0.00	7.09 (0.97) 13.70%	214.75 (7.59) 3.53%
Sand	3	204.64 (15.74) 7.69%	3.22 (2.79) 86.62%	7.59 (1.44) 18.94%	208.62 (6.66) 3.19%
Sand	6	189.99 (2.71) 1.43%	1.67 (2.89) 173.21%	9.51 (1.07) 11.24%	206.46 (9.46) 4.58%
Clay; control 1*	0	224.67	0.00	4.42	203.08
Clay; control 2*	3	202.56	0.00	10.00	206.06
Clay; control 3*	6	192.22	0.00	9.88	212.74
Anhydrous sodium sulfate; blank	0	201.04 (14.95) 7.43%	0.00	0.00	0.00

* No bacteria added

Table 6. Results of lindane recovery (%Recovery$_{Lindane}$), total DDT recovery (%Recovery$_{\Sigma DDT}$), and *p,p′*-DDT recovery (%Recovery$_{DDT}$) with their corresponding standard deviations (SD) and relative standard deviations (%RSD) for the clay and sand microcosm experiments after 0, 3, and 6 weeks of incubation. Results obtained for the control and blank experiments are also shown for comparison.

Mineral substrate	Incubation time (weeks)	%Recovery$_{Lindane}$ (SD) %RSD	%Recovery$_{\Sigma DDT}$ (SD) %RSD	%Recovery$_{DDT}$ (SD) %RSD
Clay	0	95.70 (1.41) 1.47%	98.96 (1.48) 1.50%	87.68 (0.56) 0.64%
Clay	3	96.56 (6.20) 6.42%	96.08 (2.37) 2.47%	83.69 (1.37) 1.63%
Clay	6	97.26 (3.56) 3.66%	92.29 (0.40) 0.44%	78.30 (0.35) 0.44%
Sand	0	92.31 (5.44) 5.89%	96.60 (2.94) 3.04%	93.19 (3.27) 3.51%
Sand	3	96.86 (7.48) 7.73%	96.64 (4.71) 4.87%	90.52 (2.86) 3.16%
Sand	6	98.81 (1.43) 1.45%	94.02 (4.83) 5.13%	89.57 (4.08) 4.56%
Clay; control 1*	0	97.8	90.2	88.1
Clay; control 2*	3	95.8	94.2	89.4
Clay; control 3*	6	100.0	97.1	92.3
Anhydrous sodium sulfate; blank	0	95.40 (4.27) 4.48%	–	–

* No bacteria added

concentration of *p,p′*-DDT (up to 10%) was also observed in the sand experiments, with a total absolute removal of 0.68 µmol of *p,p′*-DDT in 6 weeks. The measured *p,p′*-DDT concentrations were, however, in the same value range as concentrations measured in the abiotic control experiments (clay experiments with no bacteria added; between 203.1 and 212.7 µg/g).

A progressive increase in the concentration of *p,p′*-DDD was observed with incubation time in the clay experiments (Table 5; Figure 5), with a total production of 14.3 µg/g of *p,p′*-DDD in 6 weeks. The measured concentration of *p,p′*-DDD was also increasing in the sand experiments but to a lesser extent (9.5 µg/g in 6 weeks) and values were again within the same range as abiotic control values (between 4.4 and 10.0 µg/g). The *p,p′*-DDT standard used in this study, being 98.0% pure, may have contained 2.0% of *p,p′*-DDD as an impurity. A maximum of 5.0 µg/g of *p,p′*-DDD measured after 0, 3, and 6 weeks of incubation for both the sand and clay microcosms may have been present as an impurity rather than as a reaction product. Hence, the amount of *p,p′*-DDD actually produced during the experiments may have been <4.5 µg/g in the sand experiments compared to up to 9.3 µg/g in the clay experiments. Results obtained for the clay experiments with no bacteria added (clay controls) also indicate that a small proportion of the added DDT may have degraded through an abiotic reaction with the non-reduced clay, producing up to 5 µg/g DDD. The amount of *p,p′*-DDD measured in the clay experiments at time 0 is also relatively large (9.7 µg/g), indicating that at least 4.7 µg/g of *p,p′*-DDD must have formed rapidly at the beginning of the

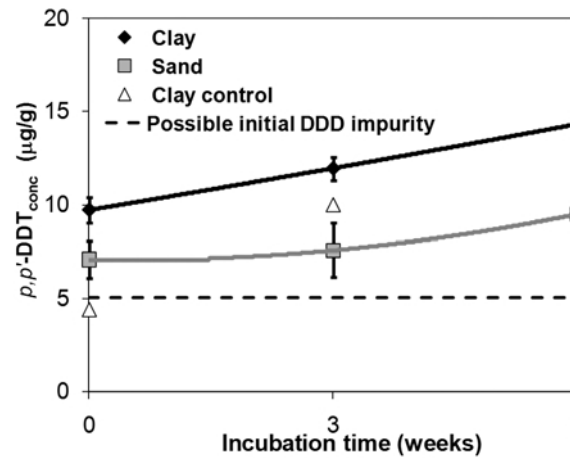

Figure 5. Results from triplicate experiments showing the measured concentration of *p,p′*-DDD for the nontronite clay and silica sand experiments after 0, 3, and 6 weeks of incubation. Concentrations measured in the clay controls (no bacteria added) are presented for comparison. The dashed line represents the amount of DDD which may be present as an impurity at the beginning of the experiment as the purity of the injected DDT was 98.0%.

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microcosm experiments, prior to the extraction of DDT and degradation products, through a biotic or abiotic reaction with the clay.

The minimal removal of DDT and DDD production in the sand microcosms, even though the micro-organism population increased and was similar to that in the clay microcosms, shows that the bacteria may have contributed little to the progressive reductive hydrogenolysis of *p,p′*-DDT to *p,p′*-DDD but that a substantial proportion of the degradation occurring during the clay experiments was promoted by the clay mineral itself. Fe^{3+} reduction in solution was either insufficient to create reducing conditions for the hydrogenolysis of DDT to DDD in the sand microcosms or structural Fe(II) in the nontronite was more reactive than Fe^{2+} adsorbed on the sand for the reductive degradation of DDT. The Fe in the nontronite would act as a 'redox mediator' (reversibly transferring electrons).

The TOC analyses have shown that the nontronite contained about four times more organic carbon than the sand. Owing to its great affinity for organic matter ($K_{oc} = 10^{5.18}$; ATSDR, 2002), *p,p′*-DDT is likely to bind to the organic matter present in the clay and sand samples. Natural organic matter often binds to the surface of clay minerals (*e.g.* Meunier, 2005). Hence, the binding of DDT to the natural organic matter present in the clay sample may have increased the availability of *p,p′*-DDT to the clay surface, therefore facilitating its reductive degradation according to the suggested mechanism (Figure 2). Further anaerobic experiments for a longer incubation time after complete removal of the organic matter and after mixing the sample obtained with various types and amounts of organic matter would allow for testing of this hypothesis.

While little or no *p,p′*-DDE was present in the abiotic controls and sand microcosm experiments, a relatively constant concentration of 13.7 to 14.7 μg/g of *p,p′*-DDE was measured in the clay microcosm experiments (Table 5). Because it was fairly constant over the 6 weeks of incubation, the *p,p′*-DDE measured probably resulted from a rapid aerobic reaction of dehydrochlorination of DDT at the beginning of the microcosm experiments, before anaerobic conditions were fully established by degassing of the headspace with O_2-free N_2 gas. Because no DDE was measured in the abiotic control experiments, the facultative aerobic bacteria *S.o.* MR-1 was probably involved in this dehydrochlorination. In addition, the very small concentration of *p,p′*-DDE measured in the sand experiments (<3.3 μg/g) compared to the clay experiments (13.7−14.7 μg/g), even though the exact same degassing procedure was applied, suggests that the nontronite may have contributed to the aerobic degradation of DDT to DDE. Further experiments with *S.o.* MR-1 under aerobic conditions are required to test these hypotheses. In particular, the pH of the suspensions must be controlled more closely to exclude any unwanted acceleration of the DDT to DDE reaction, which is known to be pH dependent (Tian *et al*., 2009).

No other metabolic products of *p,p′*-DDD, such as DDMU, were observed, indicating that either conditions in the microcosms or the timescale did not allow the formation of these other products.

ENVIRONMENTAL SIGNIFICANCE AND RECOMMENDATIONS

The results of this study have important implications in the field of environmental science, from both a biogeochemical and environmental engineering point of view. The evidence presented here confirmed that, under anaerobic conditions, Fe-bearing clay minerals have the potential to contribute to the natural attenuation of the pesticide DDT through a coupled abiotic-biotic process involving Fe within the molecular structure of the clay minerals and Fe-reducing bacteria. Because metal-reducing micro-organisms and Fe-bearing clay minerals are often abundant in soils and sediments, this kind of process is likely to play a substantial role in the reductive transformation of DDT and other redox-sensitive contaminants (*e.g.* nitroaromatics, chlorinated aliphatics, hexavalent chromium) frequently observed in contaminated soils, sediments, and aquifers. From an environmental engineering point of view, the present study also opens new avenues for the passive treatment of DDT in contaminated land in that the rate of degradation of DDT in contaminated soils, sediments, or aquifers could be enhanced through the injection of Fe-reducing chemicals or Fe-reducing bacteria and suitable nutrients to reduce Fe-bearing minerals naturally present in the contaminated area. Such *in situ* reactive zones (IRZ) are already being used for the engineered treatment of various redox-sensitive contaminants with great success (*e.g.* Suthersan and Payne, 2005). Fe-bearing clay minerals and *in situ* Fe reduction could also be used in permeable reactive systems to treat groundwater contaminated with DDT or other redox-sensitive contaminants. However, the observed reductive degradation of DDT to DDD by the bacterially reduced nontronite NAu-2 over the 6 week period (~22%) is much less than that observed using other approaches (Aislabie *et al.*, 1997). For example, the study by Guenzi and Beard (1967) found direct conversion of nearly 80% of DDT to DDD in only 4 weeks using natural soil samples incubated under anaerobic conditions. None of the evidence presented by those authors, however, establishes whether any of the mineral or organic materials present in the soil or sediment samples played any role in the degradation. Because clay minerals are common and often abundant in soils and sediments around the world, assessing the true impact of the mineral substrate *vs.* the bacterial activities in the natural attenuation of DDT and other recalcitrant contaminants is of major importance. Further work should include

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anaerobic experiments with different clay minerals and bacterial communities, possibly including bacteria isolated from soil samples used in other studies (*e.g.* Guenzi and Beard, 1967). In addition, other experiments using contaminated soil samples mixed with various amounts of clay additives could be conducted to further assess the 'redox mediator' effect of the clay minerals on the natural attenuation of chlorinated contaminants. Completely abiotic reduced controls would also allow further establishment of the role of the reduced clay minerals in degrading chlorinated contaminants.

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APPENDIX

As recommended by the U.S. EPA (US EPA, 2007b), individual relative response factors (RRF; response factor of the analyte normalized to the response factor of the internal standard) were determined for each analyte at each calibration level using equation 1 and mean RRF values were calculated using equation 2.

$$\mathrm{RRF} = \frac{A_s \times W_i}{A_i \times W_s} \quad (1)$$

with A_s = peak area of the internal standard (octafluoronaphthalene:OFN), A_i = peak area of the surrogate standard or analyte, W_i = weight of the surrogate standard or analyte in μg, and W_s = weight of the internal standard (OFN) in μg.

$$\mathrm{MeanRRF} = \frac{\Sigma \mathrm{RRF}}{n} \quad (2)$$

with n = number of RRF values.

The relative standard deviation (%RSD) was also calculated using equation 3 for each analyte to assess whether the relationship between RRF values is linear (*i.e.*, if %RSD is $\leqslant$15%).

$$\%\mathrm{RSD} = \frac{100 \times SD}{\mathrm{MeanRRF}} \quad (3)$$

with SD = standard deviation of the RRF values obtained for each dilution.

The RSD for each organic standard was <15% (Table 2), indicating that a quadratic equation was not required to fit the RRF values and that the mean RRF values were sufficiently accurate to be used in any further calculations.

The meanRRF was, therefore, used to calculate the concentration of each analyte ([i], in μg/g) in each sample using equation 4.

$$[i] = \frac{\alpha \times A_i \times W_s}{\mathrm{MeanRRF} \times A_s \times W_{solid}} \quad (4)$$

with A_s = peak area of the internal standard (OFN), A_i = peak area of the surrogate standard or analyte, W_s = weight of the internal standard (OFN) in μg, W_{Solid} = weight of sand or clay in g, MeanRRF = mean relative response factor for the surrogate standard or analyte, and α = dilution factor (*i.e.* 250).

The percent recovery was used to determine the efficiency of the extraction using the surrogate standards and was calculated using equation 5.

$$\%\mathrm{Recovery} = \frac{100 \times [i]_{measured}}{[i]_{added}} \quad (5)$$

with $[i]_{measured}$ = measured concentration of the surrogate standard or analyte i in μg/g and $[i]_{added}$ = added concentration of the surrogate standard or analyte i in μg/g.

The percent recovery was also used to assess the reliability of the measured ΣDDT concentrations for the degradation experiments using equation 6.

$$\%\mathrm{Recovery}_{\Sigma DDT} = \frac{100 \times \Sigma[\mathrm{DDT}]_{measured}}{[p,p' - DDT]_{added}} \quad (6)$$

with $\Sigma[DDT]_{measured}$ = sum of the measured concentrations for *p,p′*-DDT and all its degradation products (DDE, DDD) in μmol/g and [*p,p′*-DDT]$_{added}$ = initially added concentration of *p,p′*-DDT in μmol/g.

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