Natural Organic Matter Does Not Diminish the Mammalian Bioavailability of 2,3,7,8-tetrachlorodibenzo-p-dioxin

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Core ideas:

- Aquatic natural organic matter (NOM) was used to study the bioavailability of TCDD.
- NOM-sorbed TCDD induced hepatic *cyp1A1* mRNA expression in mice.
- NOM-sorbed TCDD suppressed humoral immune function in mice.
- NOM-sorbed TCDD manifested no reduction in bioavailability compared to the control.

Abstract:

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a toxic and persistent organic pollutant found in soils and sediments. It has been linked to several adverse health outcomes in humans and wildlife, including suppression of the immune system. TCDD is strongly sorbed to soils/sediments due to its extremely low water solubility. Presently, the bioavailability of soil/sediment-sorbed TCDD to mammals is not completely understood. Our previous studies demonstrated that TCDD adsorbed to representative inorganic geosorbents (i.e. porous silica and smectite clay) exhibited the same bioavailability to mice as TCDD dissolved in corn oil, whereas sequestration by activated carbons eliminated TCDD bioavailability. In this study, we evaluated the effects of amorphous natural organic matter (NOM), primarily in the form of aquatic humic and fulvic acids, on the mouse bioavailability of TCDD. An aqueous suspension of TCDD mixed with NOM was administered to mice via oral gavage. The relative bioavailability of TCDD was assessed by two sensitive aryl hydrocarbon receptor-mediated responses in mice: 1) hepatic induction of *cyp1A1* mRNA; and 2) suppression of immunoglobulin M (IgM) antibody-forming cell (AFC) response which is an indicator of immunotoxicity. Hepatic induction of *cyp1A1* mRNA and suppression of IgM AFC induced by TCDD were similar in the NOM-sorbed form and dissolved in corn oil, revealing no loss of bioavailability when associated with NOM. Hence, NOM-associated TCDD is as capable of suppressing humoral immunity in mice as TCDD dissolved in corn oil, indicating that NOM-sorbed TCDD is likely to fully retain its bioavailability to mammals and, by inference, humans.

Keywords: TCDD, amorphous natural organic matter, bioavailability

Introduction

Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) are groups of highly toxic chemicals with exceptionally low aqueous solubility. They were listed as persistent organic pollutants (POPs) in the 2001 Stockholm Convention with an estimated plasma half-life in humans of approximately 7 years (Pirkle et al., 1989). Substantial studies have been performed on the relationship between exposure to PCDD/Fs and ecological and human health problems, especially with TCDD. Cohort studies and animal experiments strongly indicated that unsafe and/or long-term exposure to dioxins can cause cancer, damage the immune system, cause reproductive and developmental problems, and skin conditions such as chloracne (Hinsdill et al., 1980; Assennato et al., 1989; Bertazzi et al., 1993; Li et al., 1995; Alaluusua et al., 1996; Aylward et al., 1996; Bertazzi et al., 2001; Luong et al., 2018). Concerns surrounding PCDD/Fs toxicity has caused the US EPA to consider lowering the cleanup criterion for residential soils from its current value of 1 ppb TEQ to 0.07 ppb TEQ (EPA, 2009); the State of Michigan criterion is currently 0.09 ppb TEQ (MDEQ, 2012).

PCDD/Fs are generated as byproducts from both anthropogenic activities and natural events. Although new regulatory controls and improved technologies have contributed to significantly reduced industrial emissions of dioxin-like compounds (DLCs) (approximately 90% reduction from 1987 to 2000 in the US (EPA, 2006)), their production will never cease. The majority of dioxins and DLCs are produced from waste incineration, poorly- or un-controlled combustion such as backyard barrel burning of refuse, forest and landfill fires, volcanic activities, and from industrial processes including metal operations, chemical manufacturing, and chlorine bleaching in pulp and paper mills (EPA, 2006; Kulkarni et al., 2008). Formation of dioxins through microbial activities during composting (Malloy et al., 1993), additions of Cl to phenols in soils (Hoekstra et al., 1999), photolysis (Lamparski et al., 1980) or clay-catalyzed dimerization (Gu et al., 2011) of highly chlorinated phenols, exposure of pesticides to sunlight (Holt et al., 2012), and *in situ* on ball clays (Gu et al., 2008) are also known but (likely) less significant than that from combustion and/or industrial processes. It remains unclear why there are 5000 kg/year more octachlorodibenzo-*p*-dioxin (OCDD) deposited onto world soils than that can be accounted for by known emissions (Baker and Hites, 2000).

Dioxins and furans are ubiquitous in the environment, with background totals in soils averaging ~1 ppb (ng/g), most of which is OCDD (EPA, 2007; Demond et al., 2008). Global atmospheric emissions of PCDD/Fs have been estimated at 2000-3000 kg per year (Baker and Hites, 2000).

Soils and sediments serve as the most significant reservoirs of PCDD/Fs due to their deposition from the atmosphere (Brzuzy and Hites, 1995; DuarteDavidson et al., 1997) and extremely low water solubility, which are estimated at 19 parts per trillion for TCDD and 0.23 part per trillion for OCDD (Marple et al., 1986; Oleszek-Kudlak et al., 2007). PCDD/Fs persist in soils and sediments due to their resistance and/or limited access to biodegradation and photodegradation, and low vapor pressure leading to extremely slow volatilization (Hagenmaier et al., 1992; Orazio et al., 1992; Li et al., 2012). Both laboratory and field studies indicated long-term persistence of PCDD/Fs in soils (Orazio et al., 1992; Hagenmaier et al., 1992) with half-lives ranging from 10 to 100 years (Seike et al., 2007; Young, 1983; Nauman and Schaum, 1987).

Due to their toxicity and persistence, PCDD/Fs in soils pose health risks to humans and wildlife from soil and dust ingestion, with greater concern for vulnerable groups like young children and pregnant women. Daily soil ingestion for young children was reported to be greater than 100 mg soil/d (Stanek and Calabrese, 1995). Deliberate soil ingestion, pica, has been documented during pregnancy in some poverty-stricken populations (>31% in low-income Mexican women and 65% in low-income black women) (Cooksey, 1995; Simpson et al., 2000) further increasing the risk of exposure to dioxins and DLCs through contaminated soils. General daily ingestion of contaminated soils/dusts can also expose infants as demonstrated by elevated levels of PCDD/Fs in breast milk and the umbilical cord in women residing near a highly contaminated site in Vietnam (Nghi et al., 2015; Boda et al., 2018). Wildlife animals such as zebra can ingest soils up to 3 g/kg body mass/day (Turner et al., 2013) while the Colorado mule deer has an estimated soil ingestion of approximately 30 g/d (Arthur and Alldredge, 1979). Michigan deer hunters are advised to minimize their consumption of fat from deer taken along the Tittabawassee or Saginaw rivers where contamination has been identified (MDHHS, Accessed on February 6, 2019).

In soil- and sediment-water systems, PCDD/Fs are present predominately in the sorbed state owing to their exceedingly low water solubilities. Sorption occurs via interactions with one or more of the major component geosorbents comprising these natural materials, with each potentially influencing PCDD/F bioavailability differently. Soils can be viewed as dual phase sorbents consisting broadly of organic matter and mineral matter (Chiou, 2002). Soil organic matter itself can be viewed as a dual phase sorbent consisting of both amorphous organic matter (AOM) which functions as a partition phase, and pyrogenic carbonaceous matter (PCM) which is an adsorbent (Chiou et al., 2000, 2015). As detailed below, the adsorptive affinity of

PCM for PCDD/Fs is considerably greater than that associated with partitioning into AOM (Chiou et al., 2015; Cornelissen et al., 2005). However adsorption of neutral organic contaminants (NOCs) like PCDD/Fs by PCM can be limited by its available surface area and/or pore volume. The adsorption of NOCs by PCM is a competitive process among coexisting solutes whereas partitioning into AOM is not (Chiou et al., 1979, 2015; Chiou 2002). Among mineral phases in soils, smectite clays can effectively adsorb certain classes of NOCs including dioxins (Boyd et al., 2001; Liu et al, 2009). The effective adsorption domains in the clay interlayers consist of planar hydrophobic siloxane surfaces made available by the presence of weakly hydrating exchangeable cations such as K+ and Cs+ (Boyd et al., 2001; Liu et al., 2009; Rana et al., 2009; Boyd et al., 2011a). The abundance of such sites in soils can be limited by the absence of smectite clays in certain locations and the fact that K+ and Cs+ are not typically dominant exchangeable cations. The role of clay minerals in sorption of PCDD/Fs in soils is probably not dominant unless the soil organic carbon content is very low (<0.1%) (Cheng et al., 2012). Soil organic matter (SOM), although less than 10% of the total mass in most soils, is generally considered the dominant geosorbent for NOCs including PCDD/Fs (Luthy et al., 1997). Support for the importance of SOM in controlling dioxin sorption comes from the strong correlation (r^2 ranged from 0.88 to 0.99) between PCDD/F sorption and SOM contents of soils (Brzuzy and Hites, 1995).

Bioavailability of a soil-sorbed contaminant is critical to evaluating organismal exposure, understanding risk, and predicting the feasibility of biodegradation (Council, 2003; Ren et al., 2018). While association with soils decreases the oral bioavailability of PCDD/Fs to mammals (Budinsky et al., 2008; Kimbrough et al., 2010), the mechanism for this bioavailability reduction remains unknown. Prior studies have measured bioavailability from 1 to 80% (relative to the liquid vehicle used to administer PCDD/Fs without soil) using 15 whole soils and five species of mammals (Budinsky et al., 2008; Kimbrough et al., 2010). However, the soils were only minimally characterized so it is difficult to extrapolate these results to predict PCDD/Fs bioavailability in other soils. Our contention is that PCDD/Fs bioavailability from soils would be better understood and perhaps predicted by analyzing the bioavailability of PCDD/Fs sorbed to the individual geosorbents that comprise soils. To test this hypothesis, we have measured the relative oral bioavailability in mice of the most important congener, TCDD, when adsorbed to porous silica (Kaplan et al., 2011) and to both synthetic and natural smectite clays (Boyd et al., 2011b). The TCDD adsorbed to these minerals caused similar toxicity responses as observed without the minerals, indicating that mineral-sorbed TCDD was 100% bioavailable to mice

relative to TCDD in a corn oil vehicle (Boyd et al., 2011b; Kaplan et al., 2011). By contrast, in another study the relative oral bioavailability of TCDD to mice was completely eliminated (~0% bioavailable) through adsorption to activated carbon (Boyd et al., 2017).

Activated carbon (AC) is an anthropogenic form of pyrogenic carbonaceous matter (PCM) prepared at high temperatures (Fig. 1) (Pignatello et al., 2017). AC has high surface area associated with a large internal porosity consisting of structured, polyaromatic (graphitic) surfaces (Marsh and Rodriguez-Reinoso, 2006) at which hydrophobic molecules like PCDD/Fs readily accumulate. Most PCMs form at much lower temperatures than AC, and therefore have smaller amounts of polyaromatic surface area (Pignatello et al., 2017). At the other extreme is AOM which forms naturally in soils at lower ambient temperatures. This sorptive component is viewed as a low surface area (Pennell et al., 1995; Chiou et al.,2000) bulk phase organic partition medium of intermediate polarity. Contaminant (e.g. TCDD) retention results from the solubilization of solutes into the interior network of the partition phase (Chiou et al., 1979; Chiou 2002). The extent of sorption is dependent on the solubility of the solute in this phase versus its solubility in water. Hence the retention mechanisms by AOM versus PCM are fundamentally different with the former involving contaminant dissolution in an organic partition phase and the latter solute condensation on graphitic surfaces. Sorption measurements for PCDDs show that adsorption to more aromatic PCM, such as AC, is 10-1000 times stronger than PCDD partitioning into AOM (Barring et al., 2002; Persson et al., 2002; Cornelissen et al., 2005). We hypothesize that PCDD/F sorption and bioavailability follow trends indicated in Fig. 1, with PCDD/F sorption increasing and bioavailability of sorbed PCDD/Fs decreasing along the continuum from AOM (minimum) to AC (maximum). This hypothesis indicates the possibility of PCDD/F bioavailability reduction by natural PCM, since it possesses, albeit to a lesser extent, many structural characteristics of AC (Pignatello et al., 2017).

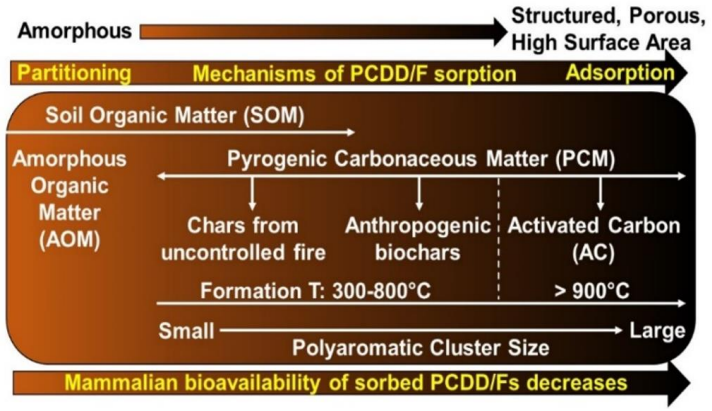

Figure 1. Schematic representation of the properties of natural soil organic matter (SOM) and pyrogenic carbonaceous matter (PCM). SOM comprised of amorphous organic matter (AOM) and naturally formed PCM is compared with anthropogenic PCM such as biochar and activated carbon (AC) regarding properties, sorption mechanisms, and bioavailability. These complex materials form a continuum as indicated.

Based on previous studies, the median PCM content (as a fraction of the total organic C content) for soils is 4% and 9% for sediments (Cornelissen et al., 2005; Pignatello et al., 2017). Additionally, only a fraction of any natural PCM will be composed of very strongly sorbing graphitic domains like those found in AC. PCDD/F sorption by SOM therefore involves, to varying extents, partitioning into AOM and adsorption to graphitic domains of PCM. We have shown that TCDD sorbed to AC was not bioavailable to mice (Boyd et al., 2017). Thus, we hypothesize that TCDD associated with AOM, the portion of SOM that lacks structured and adsorptive surfaces, will remain bioavailable to mice. The objective of the present study was to quantify that bioavailability.

In this study, reference natural organic matter (NOM) was used as a representative form of AOM. The bioavailability of NOM-sorbed TCDD to mice was evaluated with two biological endpoints that have been used as *in vivo* assays in our previous studies (Boyd et al., 2011b; Kaplan et al., 2011; Boyd et al., 2017): 1) Induction of cytochromes signaled by dioxin-ligand complexation with the aryl hydrocarbon receptor, and 2) Suppression of humoral immune responses to sheep-red-blood-cell (sRBC) antigens. The results, when combined with our previous studies on inorganic soil constituents, will provide a better understanding of how individual geosorbents contribute to the reduced bioavailability of PCDD/Fs in bulk soil.

Materials and Methods

Natural amorphous organic matter

Research grade reference natural organic matter (NOM). isolated from the Okefenokee Swamp region of the Suwannee River was obtained from the International Humic Substances Society (IHSS) and used in this study. The aquatic reference NOM was isolated using reverse osmosis (RO) as detailed previously (Serkiz and Perdue, 1990; Green et al., 2015). This avoids the conventional alkaline-extraction method used for organic matter extraction and minimizes artifacts associated with using sodium hydroxide (Lehmann and Kleber, 2015). Such RO of aquatic NOM is one of the few ways to obtain a representative NOM that is not intimately associated with soil minerals so that NOM effects can be studied independently. The IHSS reference NOM sample contains both hydrophobic and hydrophilic acids, and other soluble organic compounds present in Suwannee River. It has a 4% ash content, a 50.7% C content (Table S1) and is composed primarily of fulvic acids and humic acids (80-90% as indicated by the alkaline extraction and resin adsorption method; Paul Bloom, IHSS, personal communication).

Animals

Five to eight-week old, female pathogen-free B6C3F1 mice were purchased from Charles River Breeding Laboratories. Female mice were used because they are less aggressive, and to be consistent with our previous studies (Boyd et al., 2011b; Kaplan et al., 2011; Boyd et al., 2017; Sallach et al., 2019) (Mice were randomly divided into 9 treatment groups (5 mice/group) and housed in cages with water and feed (Purina Certified Laboratory Chow) for at least a two-week acclimation period, upon which body weights reached approximately 20 g each. Animal housing rooms were maintained on a 12:12-h light:dark cycle with temperatures between 21 to 24 °C and relative humidity between 40 to 60%. All procedures involving mice were in accordance with the Michigan State University Institutional Animal Care and Use Committee.

Preparation of treatments

A total of nine treatments were used in this study. These included TCDD sorbed to NOM suspended in water (TCDD-NOM) at three exposure concentrations, and their corresponding positive controls (TCDD-CO) comprised of equivalent amounts of TCDD dissolved in corn oil. Mice were dosed by oral gavage at either a high (10 µg/kg body mass/d), medium (1 µg/kg body

mass/d), or low (0.1 µg/kg body mass/d) TCDD level. These concentrations were selected to be consistent with the exposures used in our previous studies and are proven to induce a bioresponse (Boyd et al., 2011b; Kaplan et al., 2011; Boyd et al., 2017). Negative vehicle controls included both NOM suspension and corn oil without TCDD. In addition, a naïve group was included in which mice were neither treated (dosed) nor sensitized with sRBC.

Specifically, Kimble KIMAX glass vials (Fisher Scientific, Hampton, NH) with PTFE-faced rubber-lined caps were used to prepare TCDD-NOM suspension. To achieve desired exposure dosages, 187.5 mg of NOM was weighed in four separate glass vials. A 60 µL aliquot of TCDD dissolved in dimethyl sulfoxide (DMSO) at concentrations of 100 ppm, 10 ppm, or 1 ppm was directly added to the NOM in each glass vial resulting in TCDD-NOM mixtures at 32 µg/g, 3.2 µg/g, and 0.32 µg/g, respectively. The stock solution of 100 µg/mL TCDD in DMSO (AccuStandard Inc., New Heaven, CT) was used to prepare the high concentration TCDD-DMSO solution, and the medium and low TCDD-DMSO solutions were prepared by 10-fold serial dilutions. The TCDD-NOM mixtures were vortex mixed immediately for 10 minutes then suspended in 6 mL of ultrapure water. The final TCDD-NOM suspensions were vortexed again for 10 minutes. Settling of NOM particulates (Fig. S1) indicated that the mixtures were suspensions containing both dissolved and suspended NOM. Correspondingly, TCDD-CO was prepared by spiking 60 µL of TCDD-DMSO (100 ppm, 10 ppm, or 1 ppm) into 6 mL of corn oil. All samples were stored at room temperature for three weeks before administration to mice.

Administration of test materials and antigen sensitization of mice

The treatment groups, consisting of 5 mice per group, are summarized in Table 1 and this study followed the methods in Boyd et al. (2017). Briefly, mice in each group, except in the naïve control, were administered by oral gavage the test materials suspended in 200 µL of vehicle or vehicle only for four consecutive days. Particular care was taken to mix and resuspend NOM solids prior to each administration. On day three, each mouse (excluding naïve) received an intraperitoneal injection of 1×10^9 sheep red blood cells (sRBCs, Colorado Serum Co, Denver, CO) to initiate a humoral immune response. On the seventh day, mice were euthanized by cervical dislocation. Resected livers and spleens were collected, weighed, and homogenized. Liver tissues were stored at -70°C in TRI Reagent (Sigma-Aldrich, St. Louis, MO). Spleens were immediately processed for quantification of the anti-sRBC immunoglobulin M (IgM) antibody-forming cells (AFCs) response.

Table 1. Experimental treatment groups. Mice in each treatment group, except for the Naïve, were administered corresponding samples listed in the table below by oral gavage. Each group contained five mice.

Group	Treatment
1	Corn oil vehicle
2	Corn oil + TCDD Low (0.1 µg/kg/d)
3	Corn oil + TCDD Medium (1 µg/kg/d)
4	Corn oil + TCDD High (10 µg/kg/d)
5	NOM vehicle
6	NOM + TCDD Low (0.1 µg/kg/d)
7	NOM + TCDD Medium (1 µg/kg/d)
8	NOM + TCDD High (10 µg/kg/d)
9	Naïve

TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin; NOM: amorphous natural organic matter.

Antibody forming cell response

Enumeration of anti-sRBC IgM secreting AFCs in the spleen was performed following the Jerne plaque assay (Jerne and Nordin, 1963) as detailed previously. Briefly, diluted mouse splenocytes were mixed with 0.5% melted agar (Difco/BD), guinea pig complement (Gibco/Invitrogen), and sRBCs from the same lot used for sensitization. Each mixture was vortex mixed then poured onto a Petri dish then covered with a 24×50 mm microscope glass slide. Following overnight incubation at 37°C, the AFCs, specifically antibody-secreting plasma cells, were quantified using a Bellco plaque viewer at ×6.5 magnification. Total splenocytes from diluted samples were determined employing a ZI Coulter particle counter (Beckman Coulter, Brea, CA) and used to normalize anti-sRBC IgM AFCs/1×10^6 splenocytes.

Cyp1A1 gene expression

Cyp1A1 (Cytochrome P450 Family 1 Subfamily A Member 1), encoded by the *cyp1A1* gene, is a protein in the drug metabolizing cytochrome P450 family of enzymes. Expression of this gene is induced by AhR agonists, including TCDD, resulting in elevated levels of *cyp1A1* mRNA in the liver corresponding to increased exposure with the agonist and can be quantified by polymerase chain reaction (PCR). Homogenized livers were phase-separated with bromochlorophenol and RNA precipitation facilitated by isopropanol. Extraction, purification, and

deoxyribonuclease treatment was carried out using the Promega SV total RNA isolation system. Random primers were employed for reverse-transcription of total RNA using a high-capacity complementary deoxyribonucleic acid (cDNA) reverse-transcription kit (Applied Biosystems, Foster City, CA). A TaqMan primer/probe set for mouse *cyp1A1* (Applied Biosystems, Foster City, CA) was used to amplify the cDNA. A 7900 HT fast real-time PCR system (Applied Biosystems, Foster City, CA) was used for amplification analysis. The results were expressed as fold change and calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Statistical analysis

The mean ± SEM (standard error of mean) was determined for each treatment group. Statistical analysis on the difference of means was determined with a parametric analysis of variance. When significant differences were detected, Dunnett's two-tailed *t* test was then used to determine the difference between treatment groups and corresponding controls. For real-time PCR, statistical analysis was performed on ΔC_T values. All analyses were performed using GraphPad Prism Version 4.0a.

Results and Discussion

Natural organic matter (NOM)

This study is acontinuation of our investigation into the mammalian bioavailability of TCDD associated with the major component geosorbents in soils and sediments. The long-term goal is to determine which component(s) could account for observed reductions in PCDD/F bioavailability to a variety of organisms in contaminated field soils (Budinsky et al., 2008; Kimbrough et al., 2010). Our prior studies with component geosorbents have demonstrated that TCDD adsorbed to porous silica (Kaplan et al., 2011) or intercalated in smectite clays (Boyd et al., 2011b) fully retained its bioavailability (relative to TCDD in corn oil) when administered orally to a mammalian (mouse) model. In companion studies, activated carbon, which can be viewed as an anthropogenic end-member of pyrogenic carbonaceous matter (Fig. 1), has been shown to completely eliminate TCDD bioavailability to mice (Boyd et al., 2017; Sallach et al., 2019) and is being considered as a sorbent amendment for remediation of soils contaminated with PCDD/Fs. One remaining geosorbent type that has not been evaluated is amorphous soil organic matter, which is generally recognized as a major sorptive component for the retention of nonionic organic contaminants (NOCs) in soils and sediments (Chiou et al., 1979; Chiou et al., 2002). High surface area pyrogenic carbonaceous matter is considered the primary geosorbent for NOCs only at very low relative (aqueous) concentrations (concentration in water/water solubility) (Chiou et al., 2000).

To conduct this study, a representative sample of natural AOM was needed. Most soil organic matter presents at least two challenges in isolating AOM. First, AOM and PCM form a continuum (Fig. 1), and it is difficult to entirely separate them (Pignatello et al., 2017). Secondly, SOM also comprises a continuum of organic fragments of different molecular sizes. Smaller fragments show higher oxygen contents (lower c/o ratio) which manifests increasing polarity and stronger reactivity toward mineral surfaces (Lehmann and Kleber, 2015). As a result, pure AOM is very difficult to obtain from soils, since it may contain PCM and/or mineral-sorbed SOM. Thus, we used a natural organic matter (NOM) (IHSS reference material) isolated from the Suwannee River to represent AOM. Prior studies have demonstrated that such dissolved organic matter functions similarly to amorphous bulk phase soil organic matter as a partition phase for NOCs (Chiou et al., 1983; Chiou et al., 1986). Hence, this study advances our understanding of the bioavailability of TCDD associated with natural amorphous organic matter.

NOM-sorbed TCDD-induced hepatic cyp1A1 mRNA expression

Cyp1A1 gene expression is a mammalian biomarker for exposure of organisms to aryl hydrocarbon receptor (AhR) agonists including TCDD, which was used in this study. The AhR, which functions as a ligand activated transcription factor induces expression of the cyp1A1 gene (Denison and Nagy, 2003). Therefore, increased exposure to TCDD manifests increased AhR-mediated *cyp1A1* gene transcription. Evaluation of *cyp1A1* mRNA in the liver serves as a particularly sensitive bioassay for measuring exposure of the mouse to TCDD as it possesses multiple dioxin response elements within its promoter. The hepatic level of *cyp1A1* mRNA in mice fed both TCDD dissolved in corn oil (TCDD-CO) and TCDD associated with NOM (TCDD-NOM) varied with treatment dosages (Fig. 2). In the TCDD-CO groups, a measurable but not statistically significant increase in *cyp1A1* expression was observed at the low (0.1 µg/kg/d) dose compared to the vehicle, whereas a substantially increased ($p<0.05$) *cyp1A1* expression was determined at the medium (1 µg/kg/d) and high (10 µg/kg/d) doses. However, no statistically significant difference in *cyp1A1* expression was observed between the medium and high doses, suggesting that maximum cyp1A1 was achieved. The TCDD-NOM treatment groups showed a similar ($p>0.05$ at each TCDD dosage level) response to TCDD exposure compared to the TCDD-CO control groups. That is, compared to respective vehicles, the low TCDD dose induced a measurable but not statistically significant elevation in *cyp1A1* expression, whereas the medium and high TCDD doses induced significantly increased *cyp1A1* expression. Moreover, induction of *cyp1A1* mRNA in the mice receiving the NOM-TCDD demonstrated successful delivery of TCDD to the liver, the first target organ after gastrointestinal (GI) absorption. Hence, NOM-associated TCDD was equally bioavailable as TCDD in the corn oil vehicle, to the mammalian (mouse) model. That no other AhR agonists were included in the experimental setting is confirmed by the lack of cyp1A1 induction in either of the vehicle control groups.

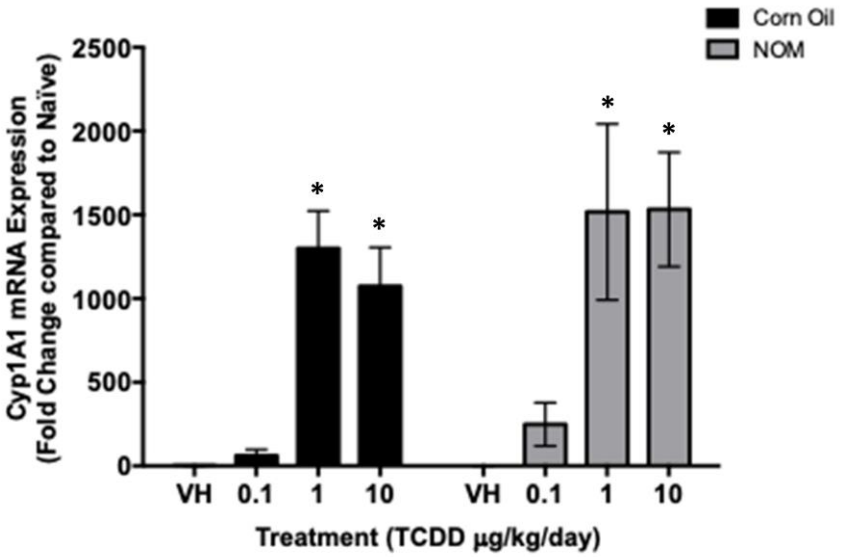

Figure 2. Liver *cyp1A1* mRNA expression induced by TCDD. TCDD dissolved in corn oil (CO) or sorbed to natural organic matter (NOM) was administered to mice at 0 (VH), 0.1 (low), 1 (medium), and 10 (high) µg/kg/d, respectively. Levels of *cyp1A1* mRNA in mice administered with each TCDD dose were compared to corresponding vehicles (VH) in terms of fold change. Expression of *cyp1A1* mRNA in mice administered NOM-sorbed TCDD were compared to that in mice receiving TCDD dissolved in corn oil for each TCDD dose. * indicates statistically significant difference ($p<0.05$) between the treated group and the corresponding VH.

The exact mechanism by which NOM-sorbed TCDD is delivered from the GI tract to the liver is unknown and beyond the scope of this study. Certainly it is plausible that desorption of TCDD from NOM occurred in the GI tract via TCDD association with gastric lipids due to its high lipophilicity (log Kow ≈ 7) (Shiu et al., 1988). What is known is that initially the dosed mass of TCDD in the TCDD-NOM treatment groups is predominately presented in the NOM-sorbed form. Prior studies of NOM in the dissolved form demonstrate that it functions as a partition phase with similar effectiveness as bulk phase soil organic matter; the sorptive effectiveness of dissolved humic acids extracted from soils were reduced only by a factor of two on a unit mass basis compared to bulk soil organic matter, i.e. $K_{om}/K_{dom} \approx 2$ (Chiou et al., 1986). In the present study, most of the NOM exists as a solid with a smaller amount presumably dissolved in water. If we assume that the TCDD partition coefficient for the NOM used here is similarly reduced by a factor of two, then estimate a K_{om} value for TCDD based on its octanol-water partition coefficient (log $K_{ow} \approx 7$) (Chiou et al., 1986), we can calculate the fractional mass of TCDD initially sorbed to NOM. Using the medium TCDD-NOM treatment (0.6 µg TCDD/187.5 mg

NOM), the fractional mass of TCDD sorbed to NOM is ca. 0.9998. Despite the fact that TCDD has almost completely partitioned into the NOM, these results show that when compared with freely available TCDD, i.e. TCDD dissolved in corn oil (TCDD-CO), the sorption of TCDD by NOM (TCDD-NOM) did not reduce the bioavailability of TCDD and hence did not reduce the exposure of the mouse to TCDD.

NOM-sorbed TCDD suppressed humoral immune function

The bioavailability of NOM-sorbed TCDD was evaluated by a second independent method, namely its ability to suppress humoral immune function in mice. Whereas induction of *cyp1A1* represents an indirect measure of TCDD exposure to AhRs in the liver, the Jerne plaque assay provides a measure of TCDD induced suppression of immune function in mice by quantifying the spleen cells (splenocytes) that produce IgM antibodies in response to a specific antigen, i.e. sheep red blood cells (sRBC) in this study. The results were thus expressed as the anti-sRBC IgM antibody-forming cell (AFC) response. In general, the IgM AFC response, using spleen cells, decreased as the dosage of TCDD increased regardless of the vehicle through which TCDD was delivered (Fig. 3). However, in the corn oil control group and the NOM treatment group at the low dose of TCDD (0.1 μg/kg/d), suppression of the IgM AFC response was either not detected (TCDD-CO group) or measurable but not statistically significant (TCDD-NOM group) ($p>0.05$). Compared to mice receiving only corn oil (VH), approximately 40% and 60% reductions ($p<0.05$) in the IgM AFC response were observed for mice administered TCDD in corn oil at the medium (1 μg/kg/d) and high (10 μg/kg/d) dosages, respectively. The reduction ($p<0.05$) in the IgM AFC responses for mice administered TCDD associated with NOM were ~70% at the medium or high doses compared to that for mice receiving only NOM (VH). Consistent with the results of *cyp1A1* expression in the liver, no statistically significant difference ($p>0.05$) in the IgM AFC response was observed between the corn oil control groups and the NOM treatment groups at each TCDD dosage level. This demonstrated a similar magnitude of suppression of the humoral immune response in mice orally administered NOM-sorbed TCDD compared to those receiving corn oil-dissolved TCDD. Additionally, suppressed humoral immune function further confirms the biodistribution of TCDD in the spleen after oral gavage.

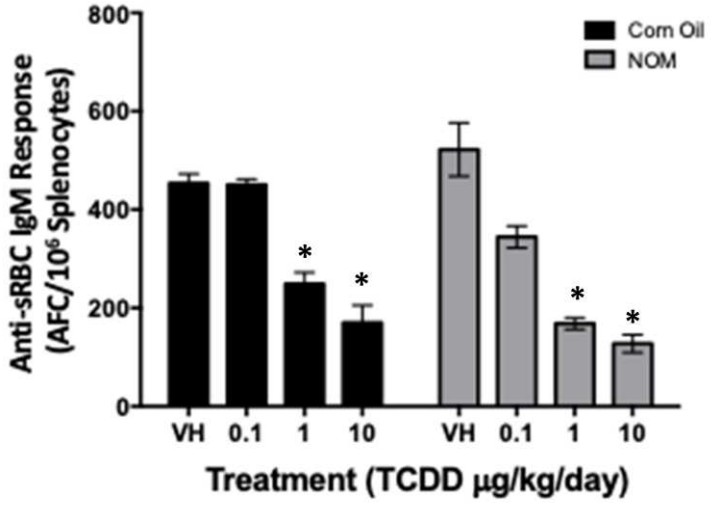

Figure 3. Suppression of the anti-sRBC IgM antibody forming cell response by TCDD. Humoral immune function of mice administered TCDD dissolved in corn oil (CO) or natural organic matter (NOM) at 0 (VH), 0.1 (low), 1 (medium), and 10 (high) µg/kg/d were evaluated through anti-sRBC IgM antibody-forming cell (AFC) response. The IgM AFC response was expressed by a bar graph. AFC response in mice administered each TCDD dosage were compared to corresponding vehicles (VH). AFC response in mice administered NOM-sorbed TCDD were compared to that in mice receiving TCDD dissolved in corn oil for each TCDD dose. * indicates statistically significant difference ($p<0.05$) between the treated group and the corresponding VH.

It is noteworthy that the low TCDD dose (0.1 µg/kg/d) had no statistically significant ($p>0.05$) effects on either *cyp1A1* mRNA expression or the IgM AFC response, which is most likely explained by this dose being below the threshold of biological activity; it is lower than the ED_{50} (effective dose) of TCDD in mice which was reported at 0.74 µg/kg/d (Kerkvliet and Brauner, 1990). The small amount of DMSO administered concurrently with NOM did not appear to affect the biological responses to NOM-sorbed TCDD, insofar as the same amount of DMSO was administered in corn oil at the low TCDD dose and no statistically significant *cyp1A1* mRNA induction or IgM AFC suppression in mice was observed.

Impact of NOM sorbed TCDD on body and organ weights

Mouse body, liver and spleen weights were measured at the termination of the study. Body weights were monitored in order to determine if TCDD exposure resulted in overt toxicity which would be indicated by a significant drop in body weight. No changes in body weight between

vehicle and TCDD dosing groups, even at the highest exposure, (Figure 4) indicates no overt toxicity. Changes in liver weight to body weight and spleen weight to body weight ratios resulting from TCDD exposure have been reported in other studies (Bryant et al., 2001; Lamb et al., 2016). However, these studies used higher TCDD exposure concentrations and measured these endpoints over a longer period of exposure. There were no significant differences between the liver weight to body weight ratios between any treatment groups (Figure S2). Spleen weight to body weight ratios for each treatment were similar between the TCDD treatments in corn oil and NOM vehicles, respectively. While a significant increase between spleen weight to body weight ratio was observed in TCDD exposed mouse groups compared to their respective vehicle control, the change in ratio (< 0.004) was negligible (Figure S2). That there were no differences between the two vehicles in either organ ratios or bodyweights supports the conclusion that TCDD complexation with NOM does not diminish its oral mammalian bioavailability.

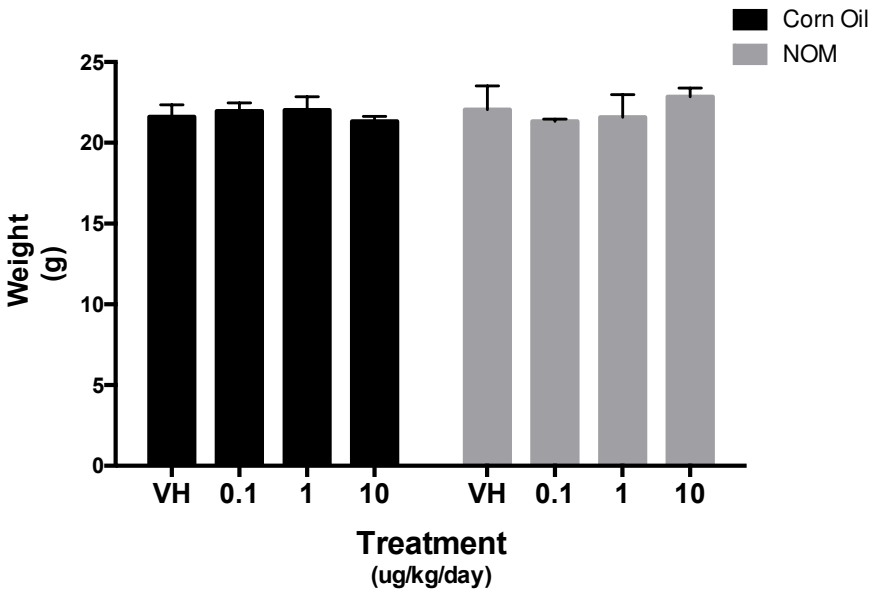

Figure 4. Mouse body weights taken at the conclusion of the feeding study (day 7) of the experiment showing no significant difference in weight, and by inference, no overt toxicity resulting from TCDD exposure in either the corn oil or NOM vehicle.

Bioavailability of NOM-sorbed TCDD was not diminished

435 The bioavailability of TCDD sorbed by natural organic matter was evaluated using both *cyp1A1*
436 induction and the IgM AFC response. The observation of induced hepatic *cyp1A1* mRNA
437 expression and suppression of humoral immune function in mice after oral gavage of NOM-
438 sorbed TCDD provide two independent biological endpoints for assessing bioavailability of
439 NOM-bound TCDD. These results are similar to those of previous studies showing that TCDD
440 sorption by other component geosorbents, i.e. porous silica Kaplan et al., 2011) and smectite
441 clays (Boyd et al., 2011b), did not reduce oral bioavailability of TCDD in mice. Thus, the major
442 finding of this study is that sorption of TCDD by NOM did not diminish its bioavailability to mice.
443 The consistency of organ impacts between NOM-sorbed TCDD and TCDD dissolved in corn oil
444 further confirmed this finding. We estimated that the fractional mass of TCDD initially sorbed to
445 the NOM administered to mice was ca. 0.9998. Only activated carbon, a high surface area
446 anthropogenic form of PCM, has been shown to sequester TCDD in a form that eliminates its
447 bioavailability to mice (Boyd et al., 2017; Sallach et al., 2019). Taken together, these studies
448 suggest that reductions in the bioavailability of PCDDs present in field soils are likely due to
449 their association with PCM (Fig. 1). Unfortunately, it is difficult to accurately assess the fraction
450 of soil organic matter that exists as PCM, making *a priori* estimates of site-specific PCDD
451 bioavailability difficult.

Conclusion

Knowledge of the bioavailability of POPs including PCDD/Fs in soils/sediments is essential for meaningful risk assessment. Understanding the mechanisms responsible for reduced mammalian bioavailability of soil-sorbed PCDDs are critical for the establishment of site-specific remediation criteria and for conceptualizing new approaches to *in situ* remediation. In the present study we showed for the first time that sorption of TCDD by amorphous natural organic matter did not manifest reductions of TCDD oral bioavailability to mice. In previous studies, sorption to representative soil minerals, i.e. porous silica (Kaplan et al., 2011) and smectite clay (Boyd et al., 2011b), similarly failed to reduce the oral bioavailability of TCDD to mice. In contrast, activated carbon strongly sequestered TCDD and eliminated its bioavailability (Boyd et al., 2017; Sallach et al., 2019). Collectively, these findings suggest that soils with a higher proportion of PCM should maximize reductions in the bioavailability of soil-sorbed PCDDs. In addition, the use of activated carbon as an *in situ* sorbent amendment to reduce the bioavailability of PCDD/Fs and similar POPs is a promising new direction in the management and remediation of large areas of contaminated soils. This remedy minimizes costs and ancillary destruction of habitat, but additional studies documenting reductions in the *mammalian bioavailability* of target contaminants are needed.

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Acknowledgement

We thank Michael D. Rizzo, Jiajun Zhou, Joseph E. Henriquez, D M Isha Olive Khan, and Lance K. Blevens from Michigan State University for their assistance with the animal related studies. This study is supported by the National Institue of Environmental Health Sciences of National Institute Health under award P42ES00491.