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

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19 **Core ideas:**

20 Aquatic natural organic matter (NOM) was used to study the bioavailability of TCDD.

21 NOM-sorbed TCDD induced hepatic *cyp1A1* mRNA expression in mice.

22 NOM-sorbed TCDD suppressed humoral immune function in mice.

23 NOM-sorbed TCDD manifested no reduction in bioavailability compared to the control.

24 **Abstract:**

25 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a toxic and persistent organic pollutant found in
26 soils and sediments. It has been linked to several adverse health outcomes in humans and
27 wildlife, including suppression of the immune system. TCDD is strongly sorbed to
28 soils/sediments due to its extremely low water solubility. Presently, the bioavailability of
29 soil/sediment-sorbed TCDD to mammals is not completely understood. Our previous studies
30 demonstrated that TCDD adsorbed to representative inorganic geosorbents (i.e. porous silica
31 and smectite clay) exhibited the same bioavailability to mice as TCDD dissolved in corn oil,
32 whereas sequestration by activated carbons eliminated TCDD bioavailability. In this study, we
33 evaluated the effects of amorphous natural organic matter (NOM), primarily in the form of
34 aquatic humic and fulvic acids, on the mouse bioavailability of TCDD. An aqueous suspension
35 of TCDD mixed with NOM was administered to mice via oral gavage. The relative bioavailability
36 of TCDD was assessed by two sensitive aryl hydrocarbon receptor-mediated responses in mice:
37 1) hepatic induction of *cyp1A1* mRNA; and 2) suppression of immunoglobulin M (IgM) antibody-
38 forming cell (AFC) response which is an indicator of immunotoxicity. Hepatic induction of
39 *cyp1A1* mRNA and suppression of IgM AFC induced by TCDD were similar in the NOM-sorbed
40 form and dissolved in corn oil, revealing no loss of bioavailability when associated with NOM.
41 Hence, NOM-associated TCDD is as capable of suppressing humoral immunity in mice as
42 TCDD dissolved in corn oil, indicating that NOM-sorbed TCDD is likely to fully retain its
43 bioavailability to mammals and, by inference, humans.

44 **Keywords:** TCDD, amorphous natural organic matter, bioavailability

45 Introduction

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

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

74 Dioxins and furans are ubiquitous in the environment, with background totals in soils averaging
75 ~1 ppb (ng/g), most of which is OCDD (EPA, 2007; Demond et al., 2008). Global atmospheric
76 emissions of PCDD/Fs have been estimated at 2000-3000 kg per year (Baker and Hites, 2000).

77 Soils and sediments serve as the most significant reservoirs of PCDD/Fs due to their deposition
78 from the atmosphere (Brzuzy and Hites, 1995; DuarteDavidson et al., 1997) and extremely low
79 water solubility, which are estimated at 19 parts per trillion for TCDD and 0.23 part per trillion for
80 OCDD (Marple et al., 1986; Oleszek-Kudlak et al., 2007). PCDD/Fs persist in soils and
81 sediments due to their resistance and/or limited access to biodegradation and
82 photodegradation, and low vapor pressure leading to extremely slow volatilization (Hagenmaier
83 et al., 1992; Orazio et al., 1992; Li et al., 2012). Both laboratory and field studies indicated long-
84 term persistence of PCDD/Fs in soils (Orazio et al., 1992; Hagenmaier et al., 1992) with half-
85 lives ranging from 10 to 100 years (Seike et al., 2007; Young, 1983; Nauman and Schaum,
86 1987).

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

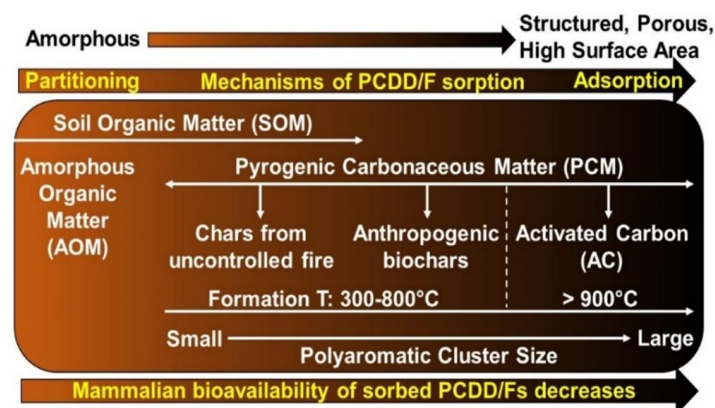
102 In soil- and sediment-water systems, PCDD/Fs are present predominately in the sorbed state
103 owing to their exceedingly low water solubilities. Sorption occurs via interactions with one or
104 more of the major component geosorbents comprising these natural materials, with each
105 potentially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

110 PCM for PCDD/Fs is considerably greater than that associated with partitioning into AOM
111 (Chiou et al., 2015; Cornelissen et al., 2005). However adsorption of neutral organic
112 contaminants (NOCs) like PCDD/Fs by PCM can be limited by its available surface area and/or
113 pore volume. The adsorption of NOCs by PCM is a competitive process among coexisting
114 solutes whereas partitioning into AOM is not (Chiou et al., 1979, 2015; Chiou 2002). Among
115 mineral phases in soils, smectite clays can effectively adsorb certain classes of NOCs including
116 dioxins (Boyd et al., 2001; Liu et al, 2009). The effective adsorption domains in the clay
117 interlayers consist of planar hydrophobic siloxane surfaces made available by the presence of
118 weakly hydrating exchangeable cations such as K⁺ and Cs⁺ (Boyd et al., 2001; Liu et al., 2009;
119 Rana et al., 2009; Boyd et al., 2011a). The abundance of such sites in soils can be limited by
120 the absence of smectite clays in certain locations and the fact that K⁺ and Cs⁺ are not typically
121 dominant exchangeable cations. The role of clay minerals in sorption of PCDD/Fs in soils is
122 probably not dominant unless the soil organic carbon content is very low (<0.1%) (Cheng et al.,
123 2012). Soil organic matter (SOM), although less than 10% of the total mass in most soils, is
124 generally considered the dominant geosorbent for NOCs including PCDD/Fs (Luthy et al.,
125 1997). Support for the importance of SOM in controlling dioxin sorption comes from the strong
126 correlation (r^2 ranged from 0.88 to 0.99) between PCDD/F sorption and SOM contents of soils
127 (Brzuzy and Hites, 1995).

128 Bioavailability of a soil-sorbed contaminant is critical to evaluating organismal exposure,
129 understanding risk, and predicting the feasibility of biodegradation (Council, 2003; Ren et al.,
130 2018). While association with soils decreases the oral bioavailability of PCDD/Fs to mammals
131 (Budinsky et al., 2008; Kimbrough et al., 2010), the mechanism for this bioavailability reduction
132 remains unknown. Prior studies have measured bioavailability from 1 to 80% (relative to the
133 liquid vehicle used to administer PCDD/Fs without soil) using 15 whole soils and five species of
134 mammals (Budinsky et al., 2008; Kimbrough et al., 2010). However, the soils were only
135 minimally characterized so it is difficult to extrapolate these results to predict PCDD/Fs
136 bioavailability in other soils. Our contention is that PCDD/Fs bioavailability from soils would be
137 better understood and perhaps predicted by analyzing the bioavailability of PCDD/Fs sorbed to
138 the individual geosorbents that comprise soils. To test this hypothesis, we have measured the
139 relative oral bioavailability in mice of the most important congener, TCDD, when adsorbed to
140 porous silica (Kaplan et al., 2011) and to both synthetic and natural smectite clays (Boyd et al.,
141 2011b). The TCDD adsorbed to these minerals caused similar toxicity responses as observed
142 without the minerals, indicating that mineral-sorbed TCDD was 100% bioavailable to mice

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

146 Activated carbon (AC) is an anthropogenic form of pyrogenic carbonaceous matter (PCM)
 147 prepared at high temperatures (Fig. 1) (Pignatello et al., 2017). AC has high surface area
 148 associated with a large internal porosity consisting of structured, polyaromatic (graphitic)
 149 surfaces (Marsh and Rodriguez-Reinoso, 2006) at which hydrophobic molecules like PCDD/Fs
 150 readily accumulate. Most PCMs form at much lower temperatures than AC, and therefore have
 151 smaller amounts of polyaromatic surface area (Pignatello et al., 2017). At the other extreme is
 152 AOM which forms naturally in soils at lower ambient temperatures. This sorptive component is
 153 viewed as a low surface area (Pennell et al., 1995; Chiou et al., 2000) bulk phase organic
 154 partition medium of intermediate polarity. Contaminant (e.g. TCDD) retention results from the
 155 solubilization of solutes into the interior network of the partition phase (Chiou et al., 1979; Chiou
 156 2002). The extent of sorption is dependent on the solubility of the solute in this phase versus its
 157 solubility in water. Hence the retention mechanisms by AOM versus PCM are fundamentally
 158 different with the former involving contaminant dissolution in an organic partition phase and the
 159 latter solute condensation on graphitic surfaces. Sorption measurements for PCDDs show that
 160 adsorption to more aromatic PCM, such as AC, is 10-1000 times stronger than PCDD
 161 partitioning into AOM (Barring et al., 2002; Persson et al., 2002; Cornelissen et al., 2005). We
 162 hypothesize that PCDD/F sorption and bioavailability follow trends indicated in Fig. 1, with
 163 PCDD/F sorption increasing and bioavailability of sorbed PCDD/Fs decreasing along the
 164 continuum from AOM (minimum) to AC (maximum). This hypothesis indicates the possibility of
 165 PCDD/F bioavailability reduction by natural PCM, since it possesses, albeit to a lesser extent,
 166 many structural characteristics of AC (Pignatello et al., 2017).



167

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

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

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

190 **Materials and Methods**

191 *Natural amorphous organic matter*

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

205 *Animals*

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

215 *Preparation of treatments*

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

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

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

239 *Administration of test materials and antigen sensitization of mice*

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

251

252 **Table 1. Experimental treatment groups.** Mice in each treatment group, except for the Naïve,
253 were administered corresponding samples listed in the table below by oral gavage. Each group
254 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

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

256 *Antibody forming cell response*

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

266 *Cyp1A1 gene expression*

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

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

280 *Statistical analysis*

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

287 **Results and Discussion**

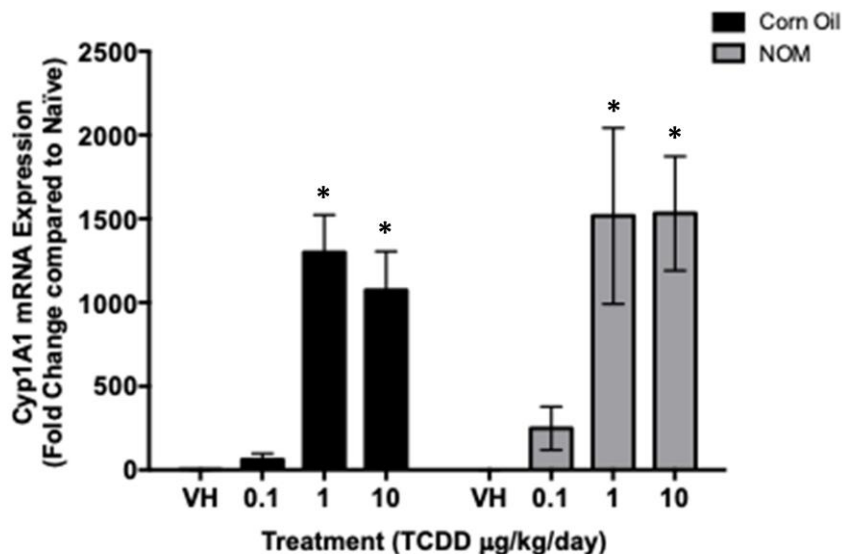
288 *Natural organic matter (NOM)*

289 This study is a continuation of our investigation into the mammalian bioavailability of TCDD
290 associated with the major component geosorbents in soils and sediments. The long-term goal is
291 to determine which component(s) could account for observed reductions in PCDD/F
292 bioavailability to a variety of organisms in contaminated field soils (Budinsky et al., 2008;
293 Kimbrough et al., 2010). Our prior studies with component geosorbents have demonstrated that
294 TCDD adsorbed to porous silica (Kaplan et al., 2011) or intercalated in smectite clays (Boyd et
295 al., 2011b) fully retained its bioavailability (relative to TCDD in corn oil) when administered orally
296 to a mammalian (mouse) model. In companion studies, activated carbon, which can be viewed
297 as an anthropogenic end-member of pyrogenic carbonaceous matter (Fig. 1), has been shown
298 to completely eliminate TCDD bioavailability to mice (Boyd et al., 2017; Sallach et al., 2019) and
299 is being considered as a sorbent amendment for remediation of soils contaminated with
300 PCDD/Fs. One remaining geosorbent type that has not been evaluated is amorphous soil
301 organic matter, which is generally recognized as a major sorptive component for the retention of
302 nonionic organic contaminants (NOCs) in soils and sediments (Chiou et al., 1979; Chiou et al.,
303 2002). High surface area pyrogenic carbonaceous matter is considered the primary geosorbent
304 for NOCs only at very low relative (aqueous) concentrations (concentration in water/water
305 solubility) (Chiou et al., 2000).

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

318 *NOM-sorbed TCDD-induced hepatic cyp1A1 mRNA expression*

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



343

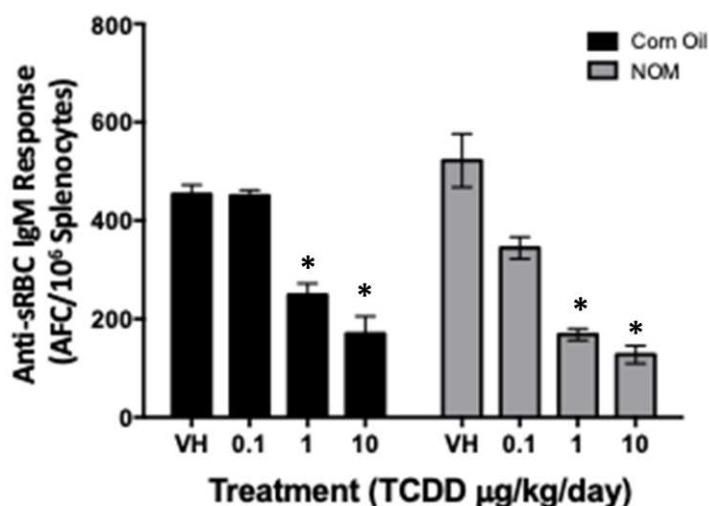
344 **Figure 2. Liver *cyp1A1* mRNA expression induced by TCDD.** TCDD dissolved in corn oil
 345 (CO) or sorbed to natural organic matter (NOM) was administered to mice at 0 (VH), 0.1 (low), 1
 346 (medium), and 10 (high) µg/kg/d, respectively. Levels of *cyp1A1* mRNA in mice administered
 347 with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

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

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

370 *NOM-sorbed TCDD suppressed humoral immune function*

371 The bioavailability of NOM-sorbed TCDD was evaluated by a second independent method,
372 namely its ability to suppress humoral immune function in mice. Whereas induction of *cyp1A1*
373 represents an indirect measure of TCDD exposure to AhRs in the liver, the Jerne plaque assay
374 provides a measure of TCDD induced suppression of immune function in mice by quantifying
375 the spleen cells (splenocytes) that produce IgM antibodies in response to a specific antigen, i.e.
376 sheep red blood cells (sRBC) in this study. The results were thus expressed as the anti-sRBC
377 IgM antibody-forming cell (AFC) response. In general, the IgM AFC response, using spleen
378 cells, decreased as the dosage of TCDD increased regardless of the vehicle through which
379 TCDD was delivered (Fig. 3). However, in the corn oil control group and the NOM treatment
380 group at the low dose of TCDD (0.1 µg/kg/d), suppression of the IgM AFC response was either
381 not detected (TCDD-CO group) or measurable but not statistically significant (TCDD-NOM
382 group) ($p>0.05$). Compared to mice receiving only corn oil (VH), approximately 40% and 60%
383 reductions ($p<0.05$) in the IgM AFC response were observed for mice administered TCDD in
384 corn oil at the medium (1 µg/kg/d) and high (10 µg/kg/d) dosages, respectively. The reduction
385 ($p<0.05$) in the IgM AFC responses for mice administered TCDD associated with NOM were
386 ~70% at the medium or high doses compared to that for mice receiving only NOM (VH).
387 Consistent with the results of *cyp1A1* expression in the liver, no statistically significant difference
388 ($p>0.05$) in the IgM AFC response was observed between the corn oil control groups and the
389 NOM treatment groups at each TCDD dosage level. This demonstrated a similar magnitude of
390 suppression of the humoral immune response in mice orally administered NOM-sorbed TCDD
391 compared to those receiving corn oil-dissolved TCDD. Additionally, suppressed humoral
392 immune function further confirms the biodistribution of TCDD in the spleen after oral gavage.



393

394 **Figure 3. Suppression of the anti-sRBC IgM antibody forming cell response by TCDD.**

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

403 It is noteworthy that the low TCDD dose (0.1 $\mu\text{g/kg/d}$) had no statistically significant ($p > 0.05$)
 404 effects on either *cyp1A1* mRNA expression or the IgM AFC response, which is most likely
 405 explained by this dose being below the threshold of biological activity; it is lower than the ED_{50}
 406 (effective dose) of TCDD in mice which was reported at 0.74 $\mu\text{g/kg/d}$ (Kerkvliet and Brauner,
 407 1990). The small amount of DMSO administered concurrently with NOM did not appear to affect
 408 the biological responses to NOM-sorbed TCDD, insofar as the same amount of DMSO was
 409 administered in corn oil at the low TCDD dose and no statistically significant *cyp1A1* mRNA
 410 induction or IgM AFC suppression in mice was observed.

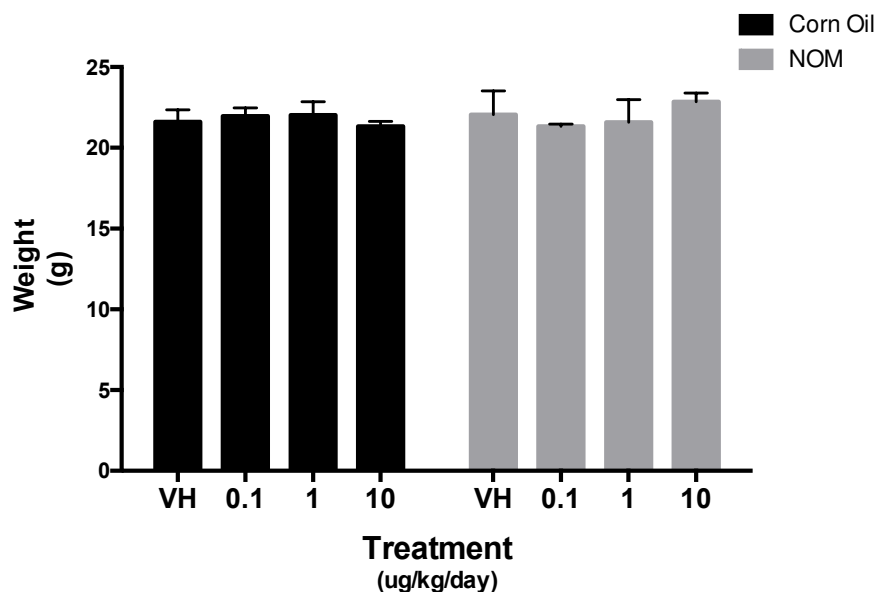
411

412 *Impact of NOM sorbed TCDD on body and organ weights*

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

416 vehicle and TCDD dosing groups, even at the highest exposure, (Figure 4) indicates no overt
417 toxicity. Changes in liver weight to body weight and spleen weight to body weight ratios resulting
418 from TCDD exposure have been reported in other studies (Bryant et al., 2001; Lamb et al.,
419 2016). However, these studies used higher TCDD exposure concentrations and measured
420 these endpoints over a longer period of exposure. There were no significant differences
421 between the liver weight to body weight ratios between any treatment groups (Figure S2).
422 Spleen weight to body weight ratios for each treatment were similar between the TCDD
423 treatments in corn oil and NOM vehicles, respectively. While a significant increase between
424 spleen weight to body weight ratio was observed in TCDD exposed mouse groups compared to
425 their respective vehicle control, the change in ratio (< 0.004) was negligible (Figure S2). That
426 there were no differences between the two vehicles in either organ ratios or bodyweights
427 supports the conclusion that TCDD complexation with NOM does not diminish its oral
428 mammalian bioavailability.

429



430

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

434 *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.

452 **Conclusion**

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

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470

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