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Article

Biological Effects of Hydrocarbon Degradation Intermediates: Is the Total Petroleum Hydrocarbon Analytical Method Adequate for Risk Assessment?

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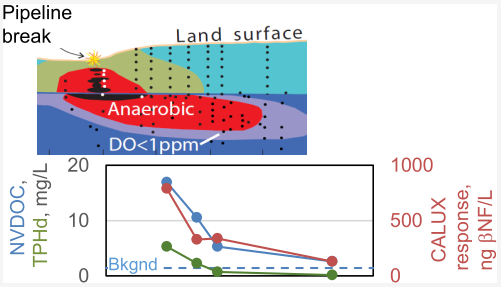
ABSTRACT: In crude oil contaminant plumes, the dissolved organic carbon (DOC) is mainly hydrocarbon degradation intermediates only partly quantified by the diesel range total petroleum hydrocarbon (TPHd) method. To understand potential biological effects of degradation intermediates, we tested three fractions of DOC: (1) solid-phase extract (HLB); (2) dichloromethane (DCM-total) extract used in TPHd; and (3) DCM extract with hydrocarbons isolated by silica gel cleanup (DCM-SGC). Bioactivity of extracts from five wells spanning a range of DOC was tested using an *in vitro* multiplex reporter system that evaluates modulation of the activity of 46 transcription factors; extracts were evaluated at concentrations equivalent to the well water samples. The aryl hydrocarbon receptor (AhR) and pregnane X receptor (PXR) transcription factors showed the greatest upregulation, with HLB exceeding DCM-total, and no upregulation in the hydrocarbon fraction (DCM-SGC). The HLB extracts were further studied with HepG2 chemically activated luciferase expression (CALUX) *in vitro* assays at nine concentrations ranging from 40 to 0.01 times the well water concentrations. Responses decreased with distance from the source but were still present at two wells without detectable hydrocarbons. Thus, our *in vitro* assay results indicate that risks associated with degradation intermediates of hydrocarbons in groundwater will be underestimated when protocols that remove these chemicals are employed.



INTRODUCTION

Monitored natural attenuation (MNA) has been accepted as a groundwater clean-up strategy for spills of petroleum hydrocarbons for the past 25 years.[1] The effectiveness of MNA for petroleum hydrocarbons was established using laboratory studies of single compounds, field observations, and database compilations of plume lengths.[1] Despite widespread evidence of biodegradation, legacy hydrocarbon groundwater contamination was still present at 126 000 sites in the United States in 2013.[2] Groundwater plumes at hydrocarbon spill sites contain both hydrocarbons and intermediate degradation products of hydrocarbons.[3−13] Many of the degradation intermediates are polar compounds comprising different chemical classes than the parent hydrocarbons and consequently they have different fate, transport, and toxicity properties.[14]

Potential hazards and/or risks posed by degradation intermediates of petroleum hydrocarbons to human health and ecological receptor communities have been the focus of several studies.[15−20] Some conclude that the risk is low;[15−17] but a number of publications present evidence that the risks of petroleum hydrocarbon intermediate degradation products to humans or ecological receptors may be greater than anticipated and in need of further characterization.[20−23] For example, two studies of bioremediation of polyaromatic hydrocarbons (PAHs) found greater toxicity associated with hydroxylated and carboxylated transformation products than with the parent PAHs.[18,19] One aspect of the issue concerns regulatory policy on the appropriate use of analytical methods to quantify total petroleum hydrocarbons (TPH) in water.[14,24] Some methods include degradation intermediates and other methods exclude these classes from quantifications and exposure assessments.

The analytical method for the aqueous concentration of TPH in the diesel range (TPHd) involves the extraction of water with a liquid solvent, typically dichloromethane (DCM) or hexane.[25] The extract is then analyzed by gas chromatography with flame ionization detection to quantify compounds that elute between the *n*-decane (*n*-C10) peak and the conclusion of the *n*-octacosane (*n*-C28) peak. Measured concentrations of TPHd reflect not only hydrocarbons but also polar compounds including some hydrocarbon degradation intermediates and natural organic matter.[8] Degradation intermediates and dissolved natural organic matter can be

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removed from the TPHd extract using silica gel cleanup (SGC), thereby isolating the hydrocarbon fraction. Currently, state regulators generally agree on risk calculation methods and approaches for aromatic and aliphatic hydrocarbons.[24,26] In contrast, approaches for risk calculations of degradation intermediates on human health are poorly constrained and controversial.[24] About half the states in the USA polled in a recent state survey responded that they do not allow removal of degradation intermediates using SGC prior to TPHd analyses.[24] The remaining states varied in their responses but typically require both SGC and non-SGC TPHd concentrations for exposure and risk evaluations.

Both methods described above (with and without SGC) are typically based on DCM extracts of groundwater samples. However, recent publications have demonstrated that the DCM extract used to quantify TPHd recovered less than half of the nonvolatile dissolved organic carbon (NVDOC) present in a crude oil plume[22,27] and a refined fuel spill.[28] The NVDOC contains the degradation intermediates as well as background DOC. Solid-phase extraction methods recover greater than 65% of the NVDOC across the range of concentrations studied at a crude oil site,[27] with the highest extraction efficiency of 92% obtained using Oasis hydrophilic−lipophilic balance (HLB) cartridges. Thus, HLB extractions are used in this study. McGuire et al.[20] performed comprehensive, *in vitro*-based bioeffects screening of HLB extracts collected from a crude oil plume. They found upregulation of molecular targets, including human nuclear receptors, that have been associated with adverse effects on development, and endocrine and liver function. However, McGuire et al.[20] did not attempt to distinguish the contributions of the hydrocarbons from the degradation intermediates in the mixture of compounds present in the groundwater plume.

The goal of this study was to establish the relative biological activity of degradation intermediates compared to C_{10}−C_{28} hydrocarbons present in a 40-year-old crude oil plume. The mixtures of compounds tested were obtained from three groundwater extraction/clean-up protocols: (1) the fraction obtained with HLB solid-phase extraction targeting a range of polar and nonpolar compounds (termed HLB); (2) the combination of hydrocarbons and degradation intermediates extracted with DCM (termed DCM-total); and (3) the hydrocarbon-only fraction obtained with DCM extraction followed by silica gel cleanup to remove degradation intermediates (termed DCM-SGC). We sampled a transect of wells in the crude oil groundwater plume spanning a range of NVDOC concentrations and degradation intermediate compounds[12] plus an unaffected reference well. Two types of *in vitro* evaluations of biological effects were performed on the extracts from the groundwater samples. The first, Attagene, measured modulation of 46 transcription factors (TF) by the mixtures of compounds in the three organic extractions.[20] Additionally, we used a human aryl hydrocarbon receptor (AhR)-based chemically activated luciferase expression (CALUX) cell bioassay to quantify activation of the AhR by HLB extracts of well water from across the range of plume NVDOC concentrations.[29]

MATERIALS AND METHODS

Site Description. The study was conducted with groundwater samples from the U.S. Geological Survey's National Crude Oil Spill Fate and Natural Attenuation Research site located near Bemidji, Minnesota (Figure 1). The site became

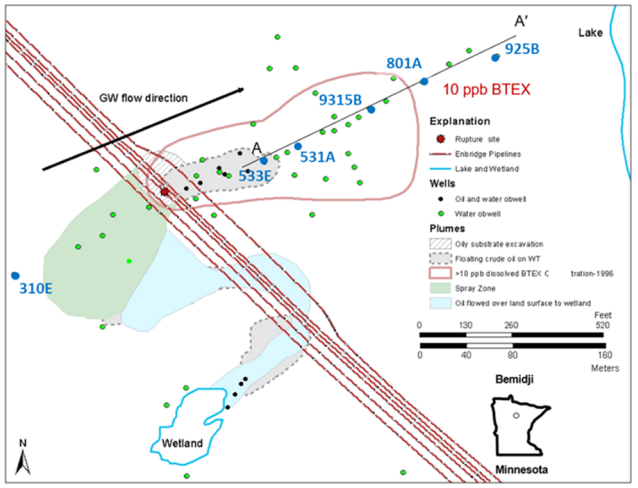

Figure 1. Site map showing locations of a crude oil source, groundwater plume, five wells sampled in the plume, and the background well unaffected by the spill (blue circles). Other features are noted in the legend.

contaminated over 40 years ago by a spill from a ruptured crude oil pipeline. Approximately, 1.7 million liters of light aliphatic crude oil was spilled, but about 75% was removed during the immediate remedial response. The remaining 25% infiltrated the glacial outwash sand and gravel aquifer within days and migrated down to the water table in three locations. Essaid et al.[30] summarized past research publications covering oil distributions, plume chemistry, microbiology, and modeling efforts at the site.

The focus of this study was the groundwater contaminant plume from the north oil body (Figure 1). The north oil body contains oil trapped in the vadose zone at saturations of 10−20% with higher oil saturations of 30−65% at the water table, located 6−8 m below the surface. The dominant microbial population carrying out biodegradation within the oil body is a methanogenic consortium[31−33] dominated by the syntrophic *d-proteobacterium Smithella* and the hydrogenotrophic *Methanoregula*.[33] A plume of nonvolatile dissolved organic carbon (NVDOC) originates in the source zone with concentrations over 30 mg NVDOC/L measured in 2016 (Figure 2). The NVDOC is mainly comprised of nonvolatile organic acids that are degradation intermediates of the crude oil compounds.[4,5,11] The NVDOC concentration migrating from the source has been documented to be over 10 times the highest concentration of benzene[22,34] and comprises the largest proportion of organic carbon in the plume. NVDOC

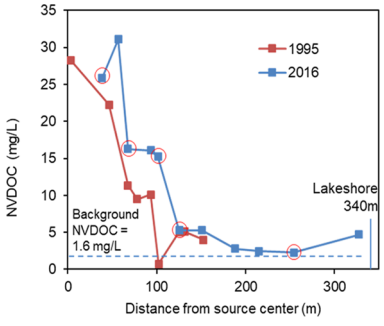

Figure 2. NVDOC concentration versus distance 1995 and 2016. The red circles denote wells sampled in 2018 for this study.

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concentrations decrease with distance from the source (north oil body) and the pool of organic carbon changes in optical character as the organic compounds are transformed in the aquifer.[12] The biotransformation in the groundwater plume is primarily coupled to iron reduction.[30] Between 1995 and 2016, the NVDOC front advanced about a meter per year as iron oxyhydroxides became depleted from the aquifer.[22,35,36] The concentration of NVDOC declines between the source and 188 m, then concentrations appear to level off between 188 m and the lakeshore (Figure 2). The concentration data and optical data suggest that there is a persistent component of the NVDOC plume that migrates to the lake.[12,22]

Sampling and Analyses. Groundwater samples were collected in June 2018 from a background (reference) well located 200 m upgradient from the source and five wells along a flowline in the plume at 39, 68, 102, 125, and 254 m downgradient from the source (Figures 1 and 2). Before sampling, at least three times the water volume in the well casing was purged and field parameters (temperature, dissolved oxygen, specific conductance, and pH) were stable.

Samples for NVDOC analyses were filtered through 0.20 μm Supor in-line filters into baked glass bottles, preserved with hydrochloric acid to a pH of <2, stored on wet ice in the field and then refrigerated at 4 °C in the laboratory until analyzed within 29 days. Concentrations of NVDOC were measured after purging with N_2 to remove inorganic carbon and volatile organics. Data presented from samples collected in 1995 were based on analysis by the persulfate wet-oxidation technique using a carbon analyzer.[37] Samples collected in 2016 and 2018 were analyzed by the high-temperature combustion technique using a Shimadzu TOC Vcsn analyzer (Shimadzu Corporation, Kyoto, Japan) as described by ref 34.

Samples for total petroleum hydrocarbons in the gasoline range (TPHg) were collected in 40 mL volatile organic analysis (VOA) vials and shipped on ice to a commercial lab. Samples were analyzed for TPHg by the EPA method SW-846 8015 using purge-and-trap gas chromatography (GC)/flame ionization detection (FID) analysis to obtain the total concentration of organics in the C_6−C_{10} range.

For TPHd analyses, two samples from each well were collected into unpreserved 1 L amber bottles and shipped on ice overnight to a commercial lab. The two samples were extracted using dichloromethane (DCM; EPA Method 3510). One sample extract was analyzed for TPHd (USEPA method 8015B). The other sample extract was treated with a silica gel cleanup (SGC) column (USEPA method 3630C) and then analyzed for TPHd (TPHd-SGC). Aliquots of the above extracts were used for the high-throughput bioassays (Attagene Inc. Morrisville, NC). To ensure compatibility with the bioassays, DCM-total and DCM-SGC extracts were dried under nitrogen gas and, once dry, reconstituted in 1 mL of dimethysulfoxide (DMSO) resulting in 1000× concentration.

A third water sample was collected from each well to perform Attagene bioassays on the organics obtained with the HLB solid-phase extraction. These samples were kept on dry ice in the field and stored at −20 °C. The samples were processed before Aug 17, 2018 (within 22 days). They were filtered using a GF/F filter (1.0 μm); 250 mL of each filtrate was concentrated using Oasis hydrophilic−lipophilic balance (HLB) 5 cm^3 200 mg cartridges (Waters, Milford, MA). The cartridges were eluted with 6 mL of methanol, followed by 6 mL of a 50:50 mixture of methanol and DCM, and brought to dryness under nitrogen gas at 20 °C. The extracts were reconstituted with 0.5 mL of dimethysulfoxide (DMSO) resulting in 500× concentration. The preparation method removes the volatile fraction. As a result, the toxicity assays performed in this study did not assess the effects of the volatile components in the plume as measured by the TPHg analyses.

Extracts generated using DCM-total, DCM-SGC, and HLB were tested in Attagene assays at 1× concentration relative to the groundwater (i.e., 1 μL of 1000× extract or 2 μL of 500× extract were added to 1 mL of growth media). Bioassays that evaluate activation of 46 molecular targets (CIS-FACTORIAL) were performed on these three extracts in duplicate (Attagene Inc. Morrisville, NC). The assay method was described by Romanov et al.[38] and deployed for the identification of molecular targets of interest in oil-contaminated groundwater samples[20] and a variety of surface waters.[39] Briefly, human hepatoma (HepG2) cells transfected with reporter constructs activated by transcription factors (TF) were used. The reporter transcript abundance was measured by isolating the produced RNA, reverse transcription, amplification, labeling, and capillary electrophoresis. Abundance data are reported as the induction by a sample of interest relative to abundance induced by a DMSO solvent control (abundance in the environmental sample was divided by abundance in solvent control). Positive control assays were performed for a subset of molecular targets (Table S2) including AhR (6-formylindolo-[3,2-*b*]carbazole) and PXR (Rifampicin). Because these novel assays are costly and the characterization of their utility is incomplete relative to some of the more traditional technologies,[40] they were primarily used to quickly screen effects on a variety of molecular targets. Once we identified the main targets of interest, we conducted an additional, widely used reporter gene assay to confirm and quantify biological activity (see below).

A fourth large-volume water sample (72−99 L) was extracted from each well with a field extraction column containing solid-phase HLB sorbent. Water samples were pumped from wells into Teflon-lined barrels, then processed through a 30 mm × 50 mm solid-phase extract (SPE), with 15 g Oasis HLB (Waters Corp., Milford, MA), with a peristaltic pump system equipped with a 100 μm fiber prefilter. Samples were either processed onsite or transported to the U.S. Geological Survey Columbia Environmental Research Center (CERC, Columbia, MO) for processing (stored at 4 °C until processed). A procedure blank (100 L Milli-Q water) was also processed through the same SPE system. The HLB sorbent material from the SPE system was transferred to a glass column (2.5 cm inner diameter) containing glass wool and sodium sulfate, the HLB wetted with ethyl acetate (EtOAc), then more sodium sulfate added to the top of the column before being eluted with 100 mL EtOAc followed by 100 mL 80:20 DCM/methyl *tert*-butyl ether (MTBE) and then 100 mL DCM. All rinses were collected in the same flask, evaporated, and brought to a final volume of 25 mL in EtOAc. Bioassay analysis for AhR activity was performed with these extracts using the HG2L7.5c1 chemically activated luciferase expression (CALUX) human hepatoma cell line[29,41] as described in the data release (https://doi.org/10.5066/P9EGYDRJ). Incubation time was 4 h post treatment of the cells to maximize activity. Cells were graciously provided by Dr. Michael S. Denison (University of California, Davis). Relative luminescence values were normalized to protein in cell lysates as described previously.[42] Results for the CALUX assay were expressed in bioanalytical equivalents (BEQ) relative to the

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activity of a well-known polycyclic aromatic hydrocarbon (PAH)-type AhR agonist (β-naphthoflavone (βNF); ng βNF/L) and represent the average of three independent experiments. BEQ values for the large-volume HLB samples were based on the quarter-maximal effective concentration (EC_{25}) value of βNF and the corresponding EC_{25} of the water sample (EC_{25} values were determined from a Hill's 4-parameter curve fit using SigmaPlot (v 14.0)).

RESULTS

Chemistry. Concentrations of NVDOC, TPHd, TPHd-SGC, and TPHg measured in the water samples were plotted versus distance from the source (Figure 3). The NVDOC concentrations in the plume were 22.7 mg/L near the source and decreased with distance but remained above the background value of 1.4 mg/L at all of the plume wells. At the two most downgradient wells (125 and 254 m), the NVDOC was 3.7 and 1.8 times the background value, respectively. TPHd-total concentrations were 31% of NVDOC near the source and decreased to 5% of NVDOC at 254 m downgradient. The concentrations of TPHd-SGC were just 1−3% of NVDOC in each sample (Figure 3). The TPHd-SGC values decreased from 0.56 mg/L near the source to 0.13 mg/L at 102 m and were below detection at 125 and 254 m. Both TPHd-total and TPHd-SGC were below detection at the background well. The volatile components in the plume measured as TPHg were 6.5 mg/L near the source, dropping to 0.4 mg/L at 102 m and below detection at 125 and 154 m.

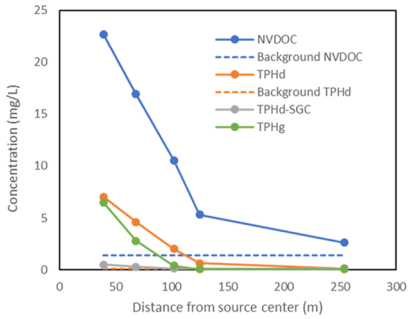

Figure 3. Concentrations of NVDOC (mg/L), diesel range total petroleum hydrocarbons (TPHd), and TPHd after polars were removed by silica gel cleanup (TPHd-SGC) measured at the five plume wells in 2018. The dashed lines show concentrations measured at the unaffected background well.

***In Vitro* Bioeffect Assays.** Biological activities of the HLB extracts from the five wells along a flowline in the plume showed upregulation for five transcription factors, especially PXR and AhR (7-fold upregulation), and exceeded those of both the DCM-total and DCM-SGC treatments (Figures 4A,B and S1). Background well HLB extracts did not upregulate any of the targets (the highest activity was 1.3-fold relative to solvent control). The PXR and AhR activity of HLB extracts increased with increasing NVDOC (Figure 4A,B). DCM-total extracts, which contain hydrocarbons and some degradation intermediates, induced biological activity higher than 2-fold for PXR and AhR, with PXR upregulated by all samples collected from the plume wells (Figures 4A and S1B), but not by the background well sample. AhR activation was the highest near the oil source (2.5-fold relative to solvent control), dropping to the assay background value by the third well (DCM-total

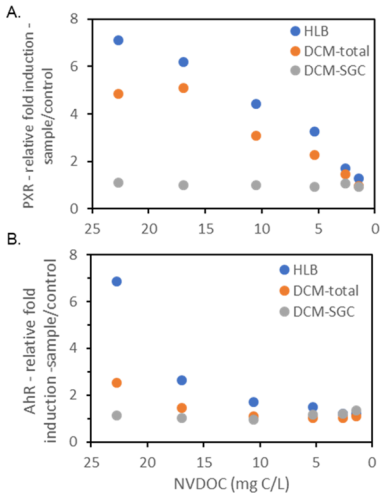

Figure 4. Induction of (A) pregnane xenobiotic receptor (PXR) and (B) aryl hydrocarbon receptor (AhR) for water samples from the five plume wells and background well. Three extracted and cleaned-up fractions were tested at concentrations equivalent to the original water samples: solid-phase extraction with Oasis HLB (HLB), liquid−liquid extraction with dichloromethane (DCM-total), and DCM extraction after removal of polar compounds with silica gel cleanup (DCM-SGC).

samples; Figures 4B and S1B). DCM-SGC extracted samples ("hydrocarbon fraction") were comparable to that of the background well; none of the samples, including the background well, induced biological activity (cutoff of 2-fold relative to solvent control was used). This was the case for all 46 transcription factors evaluated, including PXR and AhR (Figures 4A,B and S1C; see associated data release for all data ref 61). A Kendall-Tau nonparametric correlation analysis shows that there was a statistically significant association between NVDOC and AhR and PXR receptor induction by HLB and DCM-total extracts. In contrast, there was no association between NVDOC and AhR and PXR receptor induction by DCM-SGC extracts (Table S1). Although the number of samples is small, the statistically significant associations provide insight into the importance of NVDOC in driving the responses.

Transcriptional activation of AhR-mediated response in CALUX HG2L7.5c1 cells was chosen for further study because of previously observed AhR activation by HLB extracts from three wells located near the plume source[20] and the widely known responsiveness of this receptor to many hydrocarbons and hydrocarbon degradation products.[43−45] The present study examined HLB extracts from a total of four wells: two with detectable hydrocarbons, and two with degradation intermediates and no detectable hydrocarbons present based on the TPHd-SGC analyses (Figure 3 excluding the well closest to the oil source, well 533E). In Figure 5, the results for CALUX HG2L7.5c1 cells expressed as β-naphthoflavone (βNF) bioanalytical equivalents (BEQ) are plotted together with NVDOC concentrations. The AhR activity yielded average BEQs ranging from 790 ± 140 ng βNF/L near the source to 340 ± 65 and 130 ± 17 ng βNF/L for the two wells where hydrocarbons were below detection (Table 1). The activity decreased with distance and NVDOC correlates with CALUX response at the four sampled wells (n = 4; R^2 = 0.87). A background well (310E) located upgradient of the source zone yielded no activity in the CALUX assay (Table 1).

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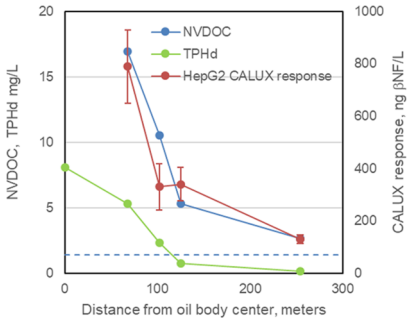

Figure 5. Values of β-naphthoflavone (βNF) bioanalytical equivalents (BEQ) as determined by CALUX human hepatoma cells (HG2L7.5c1) for four plume wells and the background well together with NVDOC and TPHd concentrations in the same wells. The dashed line is background NVDOC concentration.

Table 1. Transcriptional Activation of AhR-Mediated Response in CALUX Human Hepatoma HG2L7.5c1 Cells Expressed as β-Naphthoflavone (βNF) Bioanalytical Equivalents (BEQ)

well ID	location (m from source)	NVDOC (mg/L)	BEQ average (± se)[a], ng βNF/L water	BEQ range (min–max), ng βNF/L water
310E	background	1.42	/	/
531A	hydrocarbon plume (68 m)	16.9	790 ± 140	520–990
9315B	hydrocarbon plume (102 m)	10.5	330 ± 89	210–500
801A	oxidation product plume (125 m)	5.32	340 ± 65	270–470
925D	oxidation product plume (254 m)	2.63	130 ± 17	100–160

[a]Average of three replicate experiments; / denotes results that did not exceed 25% of the maximum response for βNF (see Figure S2).

DISCUSSION

Chemistry. NVDOC concentrations were 3–20 times the TPHd concentrations at the five wells in the plume (Figure 3). These ratios were consistent with 2010 and 2016 results from the site,[20,22] indicating they are characteristics of this crude oil plume. Thus, the TPHd analyses provided a confusing picture because they quantified both hydrocarbons and a variable fraction of the degradation intermediates. The cause was likely the use of DCM extraction for the water samples prior to TPHd analyses. Zito et al.[27] found that the mass of organic carbon obtained with DCM extractions as a percentage of NVDOC ranged from 25% near the source to 17% at 254 m downgradient. When SGC was used prior to the TPHd analyses, the reduction in mass was 97%. Moreover, beyond 125 m, the TPHd-SGC concentrations were below detection. Thus, risk calculations based on hydrocarbon concentrations obtained with TPHd and especially TPHd-SGC only account for a small portion of the NVDOC mass in this plume. Beyond about 125 m downgradient, the NVDOC concentrations in the plume leveled off at 3–5 mg/L, suggesting limited further biodegradation of NVDOC before the plume reaches the lake (Figure 2). These results are consistent with the observations in Cozzarelli et al.[34] that showed NVDOC concentrations in 2013 were 2.5–4.3 mg/L at the two furthest downgradient wells.

The very small mass of hydrocarbons remaining after SGC, compared to the total NVDOC pool, shows that the plume is dominated by degradation intermediates. Some authors have suggested that the NVDOC in the plume is comprised of a mixture of degradation intermediates and products of biosynthesis such as proteins, carbohydrates, lipid, and nucleic acids released by the aquifer microbial population.[46,47] However, data from a combination of five quantitative and qualitative analytical techniques demonstrate that the NVDOC near the source is composed of highly reduced compounds like those in the crude oil source.[48] As the components of crude oil biodegrade downgradient from the source, the NVDOC becomes more oxygenated and composition changes continuously. Principle component analysis of the combined data set shows a compositional gap between the plume wells and the unaffected background well, consistent with NVDOC originating in the oil body and differing from the background NVDOC. Accounting for the background NVDOC that migrates into the source zone, the percentage of degradation intermediates in the plume NVDOC ranges from 94% in the well closest to the source to 46% in the most downgradient well (Table 1).

Biological Effects. Previously, McGuire et al.[20] presented 2016 data for HLB extracts showing six transcriptional pathways upregulated more than 2-fold (CIS-factorial assays). The highest upregulation of biological activity was observed for PXR and AhR; biological activity decreased with distance from the source. The findings of the present study are congruent with that of McGuire et al.;[20] HLB extracts have similar profiles of biological activity and exhibited similar spatial trends—PXR decreased from 7- to 1.7-fold and AhR from 7- to 1.2-fold between 39 and 254 m downgradient. For the DCM-total extracts, this study found upregulation of only PXR and AhR with lower values compared to those for HLB (Figure 4). The use of HLB has been shown to extract an average of 92.4% of the NVDOC in the plume, the highest extraction efficiency of any methods tested.[27] Thus, the observed effects for HLB were based on the highest possible extracted concentrations of degradation intermediates. The results from this study showed that the use of DCM liquid–liquid extraction protocols missed a portion of organic carbon compounds that were associated with increased biological effects. Moreover, the use of DCM-SGC to isolate the hydrocarbons resulted in extracts with negligible activity in the Attagene assays, demonstrating that the measured biological effects in the plume were associated with the degradation intermediates. The negligible activity of the hydrocarbons in the diesel range was likely due to their low concentrations (Figure 3).

The results from the HepG2 CALUX assay showed responses throughout the plume (Figure 5). The BEQ values decreased with distance from the oil source but persisted beyond 125 m downgradient, where NVDOC values from 2016 (Figure 2) appeared to level off before the plume reached the lake. The activity was present at two wells with no detectable hydrocarbons (Figure 3), underscoring the importance of using an extraction method that captures a large fraction of the NVDOC when testing for biological effects. The downgradient AhR activity where the NVDOC concentrations were relatively constant with distance indicated that the most refractory intermediate degradation products of the petroleum hydrocarbons were agonists of the AhR and, as such, may pose risks to exposed human or ecological receptors.

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Studies are needed to assess whether the *in vitro* activity observed here translates to adverse outcomes *in vivo*. Additionally, studies are also needed to characterize the nature of the chemicals in the persistent fraction of NVDOC capable of activation of AhR pathways.

Our results can be compared with comparable assays that used βNF as the positive control to estimate BEQs. Yeast cells transfected with human AhR and its dimerization partner, the human aryl hydrocarbon receptor nuclear translocator (ARNT), have found BEQ values for river water ranging from ∼10 to 950 ng/L BEQ,[40,49] 390 to 740 ng/L in wastewater treatment plant effluent,[50] and 2 000 ng/L to 1 500 μg/L in untreated wastewater.[50−52] In one of the studies measuring BEQ of river water, the sites with the highest BEQ values (200−950 ng/L) were located within an industrial textile region of Japan,[49] an area known for containing AhR agonists due to contributions from dye industry effluents.[53] The second study examining BEQ values in river water similarly found the highest BEQ values (approximately 50−130 ng/L) to be located within an industrialized region.[40] The literature BEQ values from waters subject to industrial influence were like those obtained in this study.

Others have suggested degradation intermediates continue to biodegrade[54] and are unlikely to pose a health risk.[16,17] The results presented here suggest that the intermediate degradation products are responsible for the observed effects near the source and persist well beyond where hydrocarbons were detected. In the most downgradient well, the AhR activity was 130 ± 17 ng βNF/L (925D; Table 1), where concentrations of NVDOC were less than twice those in the background aquifer. Humic substances are known to activate AhR pathways at concentrations of 5 mg/L or greater,[55] and AhR activity was documented in surface water draining a wetland with high DOC (15.9 mg/L).[39] Thus, we examined the effect of the native NVDOC extracted from the background well at equivalent concentrations to those measured in the plume. The AhR responses at the range of tested concentrations are illustrated in Figure S2A,B. The activity of the background NVDOC was much lower than that for similar concentrations as in the plume (Figure S2B). Furthermore, there was no response of the background NVDOC at the concentration found in the background well (1.42 mg/L). This tells us that AhR ligands capable of transcriptional activation of AhR pathways were present in the downgradient portions of the plume and were caused by the intermediate degradation products and not the native aquifer NVDOC.

PXR plays a role in the regulation of hepatic glucose and lipid metabolism, but its role in xenobiotic sensing and metabolism and disposition was discovered first and is thus better characterized.[56,57] The normal physiological function of the AhR is not fully elucidated, but there is growing evidence that it plays a role in neurogenesis, hematopoiesis, and cardyomyogenesis.[58] The best understood role of AhR is in the metabolism of xenobiotic compounds; its activation can be indicative of an exposure to a foreign chemical. Activation of AhR-mediated pathways does not always lead to adverse health outcomes. However, it has been demonstrated that activation of these pathways over a period of time and over a threshold amount leads to adverse health outcomes in vertebrates.[52] Risk is proportional to activation of the AhR pathways, when that activation occurs over a threshold. Activation of PXR and AhR responses in the cell bioassays was indicative of chemicals being present in the groundwater samples at great enough concentrations to initiate the cascade of events known to be associated with these receptors. However, it is not known if the chemicals capable of activating these pathways were present at amounts significant enough to cause adverse outcomes in whole animals. Further studies are required to characterize the exact nature of the chemicals responsible for PXR and AhR activation and to determine if those chemicals are present at concentrations sufficient to cause adverse effects in organisms that might be exposed. These studies should evaluate the potential for groundwater samples along the plume, to cause adverse outcomes consistent with AhR mechanisms. Developing fish embryos would be an appropriate model for future studies, owing to their known sensitivities to both PAHs and their hydroxylated metabolites.[53]

Implications. The finding that TPHd analyses capture only a fraction of degradation products has been replicated at a second crude oil spill.[22] In a study of five fuel terminal spills, Zemo et al.[16] reported TPHd and TPHd with SGC concentrations for 22 groundwater samples. An analysis of these data reveals that polar compounds comprised an average of 77% of TPHd concentrations in the source zones and 71% in downgradient wells where hydrocarbons were below detection (see Table 4 in Zemo et al.[16]). The authors did not address or discuss the possibility that additional polar compounds might be present in these plumes that were not captured with the TPHd method.

Two alternative approaches for evaluating the risk of degradation intermediates on human health were included in the recently published "Total Petroleum Hydrocarbons (TPH) Risk Evaluation at Petroleum-Contaminated Sites."[24] The first approach assumes degradation intermediates are present in the same proportions as aromatics and aliphatics in the spilled fuel or oil, and those degradation intermediates have equal toxicity.[24] The second approach is based on a series of studies examining the polar compounds in DCM extracts.[15−17] Over 760 polar compounds were identified but not quantified.[15] The identified polar compounds were grouped into structural classes, and toxicity factors for representative compounds in each class were used to estimate the potency for the entire class.[24] The first method is problematic because it assumes that degradation intermediates have the same toxicity as parent compounds and are present at the same concentrations, even though they have different properties. Moreover, recent studies have found greater toxicity in PAH degradation products than for the parent hydrocarbons.[18,19] The second method is limited because it assumes toxicity factors for single compounds are adequate to represent a class of compounds.[21] More importantly, it is based on the fraction that is extracted with DCM (DCM-total), which recovers one-third or less of the degradation intermediates present at this site.

More information is needed on the composition of oil spill degradation intermediates and their potential risks to disrupt biological processes in both human and ecological receptors. For example, oxygenated PAHs cause acute toxic stress in a variety of aquatic organisms and both cytotoxicity and oxidative stress in mammalian cells.[59] A range of adverse effects on neurobehavior and development were observed in zebrafish embryos exposed to approximately 95 different PAH derivatives, and many of these derivatives induced CYP1A in the embryos.[60] Additionally, these oil spill degradation intermediates are often more polar than the parent compounds and move more readily through soil and water, which can increase potential for transport to a greater number of

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receptors. Our findings indicated that the intermediate degradation products from oil spills in groundwater have biological activity and can persist. Thus, sampling and analysis methods (including extraction and clean-up protocols) that exclude or under-represent the contribution of intermediate degradation products of oil may underestimate risks from these chemicals.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02220.

Results of Kendall-Tau nonparametric correlation analyses between NVDOC and AhR and PXR induction; positive control compounds used for a subset of molecular targets; results of bioassays; molecular targets with greater than 1.5-fold induction relative to abundance induced by a DMSO solvent control; the response of CALUX human hepatoma HG2L7.5c1 cells to dilutions of HLB extracts presented as water equivalents and nonvolatile dissolved organic carbon (PDF)

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Notes

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■ REFERENCES

(1) NRC. *Natural Attenuation for Groundwater Remediation*; The National Academies Press: Washington, DC, 2000; p 292.

(2) NRC. *Alternatives for Managing the Nation's Complex Contaminated Groundwater Sites*; The National Academies Press: Washington, DC, 2013; p 422.

(3) Cozzarelli, I. M.; Baedecker, M. J.; Eganhouse, R. P. Transformation of monoaromatic hydrocarbons to organic acids in anoxic groundwater environment. *Environ. Geol. Water Sci.* **1990**, *16*, 135−141.

(4) Eganhouse, R. P.; Baedecker, M. J.; Cozzarelli, I. M.; Aiken, G. R.; Thorn, K. A.; Dorsey, T. F. Crude oil in a shallow sand and gravel aquifer-II. Organic geochemistry. *Appl. Geochem.* **1993**, *8*, 551−567.

(5) Thorn, K. A.; Aiken, G. R. Biodegradation of crude oil into nonvolatile organic acids in a contaminated aquifer near Bemidji, Minnesota. *Org. Geochem.* **1998**, *29*, 909−931.

(6) Elshahed, M. S.; Gieg, L. M.; McInerney, M. J.; Suflita, J. M. Signature Metabolites Attesting to the In Situ Attenuation of Alkylbenzenes in Anaerobic Environments. *Environ. Sci. Technol.* **2001**, *35*, 682−689.

(7) Gieg, L. M.; Sulflita, J. M. Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. *Environ. Sci. Technol.* **2002**, *36*, 3755−3762.

(8) Zemo, D. A.; Foote, G. R. The technical case for eliminating the use of the TPH analysis in assessing and regulating dissolved petroleum hydrocarbons in ground water. *Groundwater Monit. Rem.* **2003**, *23*, 95−104.

(9) Young, L. Y.; Phelps, C. D. Metabolic Biomarkers for Monitoring in Situ Anaerobic Hydrocarbon Degradation. *Environ. Health Perspect.* **2005**, *113*, 62−67.

(10) Parisi, V. A.; Brubaker, G. R.; Zenker, M. J.; Prince, R. C.; Gieg, L. M.; da Silva, M. L. B.; Alvarez, P. J. J.; Suflita, J. M. Field metabolomics and laboratory assessments of anaerobic intrinsic bioremediation of hydrocarbons at a petroleum-contaminated site. *Microb. Biotechnol.* **2009**, *2*, 202−212.

(11) Islam, A.; Ahmed, A.; Hur, M.; Thorn, K. A.; Kim, S. Molecular-level evidence provided by ultrahigh resolution masss-pectrometry for oil-derived doc in groundwater at Bemidji, Minnesota. *J. Hazard. Mater.* **2016**, *320*, 123−132.

(12) Podgorski, D. C.; Zito, P.; McGuire, J. T.; Martinovic-Weigelt, D.; Cozzarelli, I. M.; Bekins, B. A.; Spencer, R. G. M. Examining Natural Attenuation and Acute Toxicity of Petroleum-Derived Dissolved Organic Matter with Optical Spectroscopy. *Environ. Sci. Technol.* **2018**, *52*, 6157−6166.

(13) Mackay, D.; Paradis, C.; Buscheck, T.; Daniels, E.; Hathaway, E.; Sieyes, N. D.; Rasa, E.; Schmidt, R.; Peng, J. Methods to Estimate Source Zone Depletion of Fuel Releases by Groundwater Flow. *Groundwater Monit. Rem.* **2018**, *38*, 26−41.

(14) Scofield, R.; Hoang, T. State of the Practice: Risk Assessment and Management of Metabolites and Degradation Products from Total Petroleum Hydrocarbons. In *Eleventh (11th) International Conference on the Remediation of Chlorinated and Recalcitrant Compounds*, Battelle, Palm Springs, CA, **2018**.

(15) Mohler, R. E.; O'Reilly, K. T.; Zemo, D. A.; Tiwary, A. K.; Magaw, R. I.; Synowiec, K. A. Non-Targeted Analysis of Petroleum Metabolites in Groundwater Using GCxGC-TOFMS. *Environ. Sci. Technol.* **2013**, *47*, 10471−10476.

(16) Zemo, D. A.; O'Reilly, K. T.; Mohler, R. E.; Tiwary, A. K.; Magaw, R. I.; Synowiec, K. A. Nature and Estimated Human Toxicity of Polar Metabolite Mixtures in Groundwater Quantified as TPHd/DRO at Biodegrading Fuel Release Sites. *Groundwater Monit. Rem.* **2013**, *33*, 44−56.

(17) O'Reilly, K. T.; Mohler, R. E.; Zemo, D. A.; Ahn, S.; Tiwary, A. K.; Magaw, R. I.; Devine, C. E.; Synowiec, K. A. Identification of ester metabolites from petroleum hydrocarbon biodegradation in groundwater using GCxGC-TOFMS. *Environ. Toxicol. Chem.* **2015**, *34*, 1959−1961.

(18) Chibwe, L.; Geier, M. C.; Nakamura, J.; Tanguay, R. L.; Aitken, M. D.; Simonich, S. L. M. Aerobic Bioremediation of PAH

Contaminated Soil Results in Increased Genotoxicity and Developmental Toxicity. *Environ. Sci. Technol.* **2015**, *49*, 13889−13898.
(19) Schrlau, J. E.; Kramer, A. L.; Chlebowski, A.; Truong, L.; Tanguay, R. L.; Simonich, S. L. M.; Semprini, L. Formation of Developmentally Toxic Phenanthrene Metabolite Mixtures by Mycobacterium sp. ELW1. *Environ. Sci. Technol.* **2017**, *51*, 8569−8578.
(20) McGuire, J. T.; Cozzarelli, I. M.; Bekins, B. A.; Link, H.; Martinović-Weigelt, D. Toxicity Assessment of Groundwater Contaminated by Petroleum Hydrocarbons at a Well-Characterized, Aged, Crude Oil Release Site. *Environ. Sci. Technol.* **2018**, *52*, 12172−12178.
(21) Brewer, R. C.; Hellmann-Blumberg, U. Nature and Estimated Human Toxicity of Polar Metabolite Mixtures in Groundwater Quantified as TPHd/DRO at Biodegrading Fuel Release Sites. *Groundwater Monit. Rem.* **2014**, *34*, 24−25.
(22) Bekins, B. A.; Cozzarelli, I. M.; Erickson, M. L.; Steenson, R. A.; Thorn, K. A. Crude Oil Metabolites in Groundwater at Two Spill Sites. *Groundwater* **2016**, *54*, 681−691.
(23) Steenson, R. A.; Hellman-Blumberg, U.; Elias, D.; Brown, K.; Fry, N.; Naugle, A.; Meiller, L.; Prowell, C.San Francisco Bay Regional Water Quality Control Board. Technical Resource Document: *Petroleum Metabolites Literature Review and Assessment Framework*; California Environmental Protection Agency, Regional Water Board. June 27, 2016.
(24) ITRC. TPH Risk Evaluation at Petroleum-Contaminated Sites. https://tphrisk-1.itrcweb.org/ (accessed November 13, 2018).
(25) *SW-846 Test Method 3510C: Separatory Funnel Liquid−Liquid Extraction*; Environmental Protection Agency: Washington, DC, 1996.
(26) MADEP. *Updated Petroleum Hydrocarbon Fraction Toxicity Values for the VPH/EPH/APH Methodology*; Massachusetts Department of Environmental Protection: Boston, MA, 2003.
(27) Zito, P.; Ghannam, R.; Bekins, B. A.; Podgorski, D. C. Examining the Extraction Efficiency of Petroleum-Derived Dissolved Organic Matter in Contaminated Groundwater Plumes. *Groundwater Monit. Rem.* **2019**, *39*, 25−31.
(28) Mackay, D.; Paradis, C.; Buscheck, T.; Daniels, E.; Hathaway, E.; Sieyes, N. D.; Rasa, E.; Schmidt, R.; Peng, J. Methods to Estimate Source Zone Depletion of Fuel Releases by Groundwater Flow. *Groundwater Monit. Rem.* **2018**, *38*, 26−41.
(29) Brennan, J. C.; He, G.; Tsutsumi, T.; Zhao, J.; Wirth, E.; Fulton, M. H.; Denison, M. S. Development of Species-Specific Ah Receptor-Responsive Third Generation CALUX Cell Lines with Enhanced Responsiveness and Improved Detection Limits. *Environ. Sci. Technol.* **2015**, *49*, 11903−11912.
(30) Essaid, H. I.; Bekins, B. A.; Herkelrath, W. N.; Delin, G. N. Crude Oil at the Bemidji Site: 25 Years of Monitoring, Modeling, and Understanding. *Groundwater* **2011**, *49*, 706−726.
(31) Bekins, B. A.; Godsy, E. M.; Warren, E. Distribution of microbial physiologic types in an aquifer contaminated by crude oil. *Microb. Ecol.* **1999**, *37*, 263−275.
(32) Fahrenfeld, N.; Cozzarelli, I. M.; Bailey, Z.; Pruden, A. Insights into Biodegradation Through Depth-Resolved Microbial Community Functional and Structural Profiling of a Crude-Oil Contaminant Plume. *Microb. Ecol.* **2014**, *68*, 453−462.
(33) Beaver, C. L.; Williams, A. E.; Atekwana, E. A.; Mewafy, F. M.; Aal, G. A.; Slater, L. D.; Rossbach, S. Microbial Communities Associated with Zones of Elevated Magnetic Susceptibility in Hydrocarbon-Contaminated Sediments. *Geomicrobiol. J.* **2016**, *33*, 441−452.
(34) Cozzarelli, I. M.; Schreiber, M. E.; Erickson, M. L.; Ziegler, B. A. Arsenic Cycling in Hydrocarbon Plumes: Secondary Effects of Natural Attenuation. *Groundwater* **2016**, *54*, 35−45.
(35) Amos, R. T.; Bekins, B. A.; Cozzarelli, I. M.; Voytek, M. A.; Kirshtein, J. D.; Jones, E. J. P.; Blowes, D. W. Evidence for iron-mediated anaerobic methane oxidation in a crude oil-contaminated aquifer. *Geobiology* **2012**, *10*, 506−517.
(36) Ng, G.-H. C.; Bekins, B. A.; Cozzarelli, I. M.; Baedecker, M. J.; Bennett, P. C.; Amos, R. T.; Herkelrath, W. N. Reactive transport modeling of geochemical controls on secondary water quality impacts at a crude oil spill site near Bemidji, MN. *Water Resour. Res.* **2015**, *51*, 4156−4183.
(37) Baedecker, M. J.; Cozzarelli, I. M. The Determination and Fate of Unstable Constituents in Contaminated Ground Water. In *Ground-Water Contamination and Analysis at Hazardous Waste Sites*; Lesage, S.; Jackson, R. E., Eds.; Marcel Dekker: New York, 1992; pp 425−462.
(38) Romanov, S.; Medvedev, A.; Gambarian, M.; Poltoratskaya, N.; Moeser, M.; Medvedeva, L.; Gambarian, M.; Diatchenko, L.; Makarov, S. Homogeneous reporter system enables quantitative functional assessment of multiple transcription factors. *Nature Methods* **2008**, *5*, 253−260.
(39) Blackwell, B. R.; Ankley, G. T.; Bradley, P. M.; Houck, K. A.; Makarov, S. S.; Medvedev, A. V.; Swintek, J.; Villeneuve, D. L. Potential toxicity of complex mixtures in surface waters from a nationwide survey of United States streams: identifying in vitro bioactivities and causative chemicals. *Environ. Sci. Technol.* **2019**, *53*, 973−983.
(40) Kawanishi, M.; Kondo, M.; Shiizaki, K.; Chu, W. L.; Terasoma, Y.; Yagi, T. Construction of a reporter yeast strain to detect estrogen receptor signaling through aryl hydrocarbon receptor activation. *Environ. Sci. Technol.* **2008**, *42*, 6897−6902.
(41) National Center for Biotechnology Information (NCBI). PubChem Database. Source=824, AID=743122. https://pubchem.ncbi.nlm.nih.gov/bioassay/743122 (accessed on Jan 8, 2020).
(42) Brennan, J. C.; Tillitt, D. E. Development of a dual luciferase activity and fluorescamine protein assay adapted to a 384 micro-well plate format: Reducing variability in human luciferase transactivation cell lines aimed at endocrine active substances. *Toxicol. In Vitro* **2018**, *47*, 18−25.
(43) Denison, M. S.; Pandini, A.; Nagy, S. R.; Baldwin, E. P.; Bonati, L. Ligand binding and activation of the Ah receptor. *Chem.-Biol. Interact.* **2002**, *141*, 3−24.
(44) Knecht, A. L.; Goodale, B. C.; Truong, L.; Simonich, M. T.; Swanson, A. J.; Matzke, M. M.; Anderson, K. A.; Waters, K. M.; Tanguay, R. L. Comparative developmental toxicity of environmentally relevant oxygenated PAHs. *Toxicol. Appl. Pharmacol.* **2013**, *271*, 266−275.
(45) Fent, K. Ecotoxicological effects at contaminated sites. *Toxicology* **2004**, *205*, 223−240.
(46) Mohler, R. E.; Ahn, S.; O'Reilly, K.; Zemo, D. A.; Espino Devine, C.; Magaw, R.; Sihota, N. Towards comprehensive analysis of oxygen containing organic compounds in groundwater at a crude oil spill site using GC×GC-TOFMS and Orbitrap ESI-MS. *Chemosphere* **2020**, *244*, 125504.
(47) O'Reilly, K. T.; Mohler, R. E.; Zemo, D. A.; Ahn, S.; Magaw, R. I.; Espino Devine, C. Oxygen-Containing Compounds Identified in Groundwater from Fuel Release Sites Using GCxGC-TOF-MS. *Groundwater Monit. Rem.* **2019**, *39*, 32−40.
(48) Podgorski, D. C.; Zito, P.; Smith, D. F.; Cao, X.; Schmidt-Rohr, K.; Wagner, S.; Stubbins, A.; Cozzarelli, I. M.; Bekins, B. A.; Spencer, R. G. M. *Biodegradation of a Petroleum-Derived Groundwater Plume Reveals the Compositional Continuum of Dissolved Organic Matter*, Fifth International Conference of CIS IHSS on Humic Innovative Technologies (HIT-2019); Book of Abstracts: Moscow, Russia, 2019.
(49) Terasaki, M.; Yasuda, M.; Makino, M.; Shimoi, K. Aryl hydrocarbon receptor potency of chlorinated parabens in the aquatic environment. *Environ. Sci.: Water Res. Technol.* **2015**, *1*, 375−382.
(50) Stalter, D.; Magdeburg, A.; Wagner, M.; Oehlmann, J. Ozonation and activated carbon treatment of sewage effluents: Removal of endocrine activity and cytotoxicity. *Water Res.* **2011**, *45*, 1015−1024.
(51) Prasse, C.; Stalter, D.; Schulte-Oehlmann, U.; Oehlmann, J.; Ternes, T. A. Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies. *Water Res.* **2015**, *87*, 237−270.
(52) Justo, A.; Gonzalez, O.; Acena, J.; Mita, L.; Casado, M.; Perez, S.; Pina, B.; Sans, C.; Barcelo, D.; Esplugas, S. Application of bioassay

https://dx.doi.org/10.1021/acs.est.0c02220
Environ. Sci. Technol. 2020, 54, 11396−11404

panel for assessing the impact of advanced oxidation processes on the treatment of reverse osmosis brine. *J. Chem. Technol. Biotechnol.* **2014**, *89*, 1168−1174.
(53) Chou, P. H.; Matsui, S.; Misaki, K.; Matsuda, T. Isolation and identification of xenobiotic aryl hydrocarbon receptor ligands in dyeing wastewater. *Environ. Sci. Technol.* **2007**, *41*, 652−657.
(54) Zemo, D. A.; O'Reilly, K. T.; Mohler, R. E.; Magaw, R. I.; Espino Devine, C.; Ahn, S.; Tiwary, A. K. Life cycle of petroleum biodegradation metabolite plumes, and implications for risk management at fuel release sites. *Integr. Environ. Assess. Manage.* **2017**, *13*, 714−727.
(55) Bittner, M.; Janošek, J.; Hilscherová, K.; Giesy, J.; Holoubek, I.; Bláha, L. Activation of Ah receptor by pure humic acids. *Environ. Toxicol.* **2006**, *21*, 338−342.
(56) Hakkola, J.; Rysä, J.; Hukkanen, J. Regulation of hepatic energy metabolism by the nuclear receptor PXR. *Biochim. Biophys. Acta, Gene Regul. Mech.* **2016**, *1859*, 1072−1082.
(57) Mackowiak, B.; Hodge, J.; Stern, S.; Wang, H. The Roles of Xenobiotic Receptors: Beyond Chemical Disposition. *Drug Metab. Dispos.* **2018**, *46*, 1361−1371.
(58) Nebert, D. W. Aryl hydrocarbon receptor (AHR): "pioneer member" of the basic-helix/loop/helix per-Arnt-sim (bHLH/PAS) family of "sensors" of foreign and endogenous signals. *Prog. Lipid Res.* **2017**, *67*, 38−57.
(59) Lundstedt, S.; White, P. A.; Lemieux, C. L.; Lynes, K. D.; Lambert, L. B.; Oberg, L.; Haglund, P.; Tysklind, M. Sources, fate, and toxic hazards of oxygenated polycyclic aromatic hydrocarbons (PAHs) at PAH-contaminated sites. *Ambio J. Hum. Environ.* **2007**, *36*, 475−485.
(60) Geier, M. C.; Chlebowski, A. C.; Truong, L.; Simonich, S. L. M.; Anderson, K. A.; Tanguay, R. L. Comparative developmental toxicity of a comprehensive suite of polycyclic aromatic hydrocarbons. *Arch. Toxicol.* **2018**, *92*, 571−586.
(61) Bekins, B. A.; Brennan, J. C.; Tillitt, D. E.; Cozzarelli, I. M.; Illig, J. M.; Martinović-Weigelt, D. *Dissolved Organic Carbon, Total Petroleum Hydrocarbons and Toxicity Assay Results for Bemidji, MN (2018)*; U.S. Geological Survey Data Release. https://doi.org/10.5066/P9EGYDRJ(July 30, 2020).

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Environ. Sci. Technol. 2020, 54, 11396−11404