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Genotoxicity and Endocrine-Disruption Potentials of Sediment near an Oil Spill Site: Two Years after the *Hebei Spirit* Oil Spill

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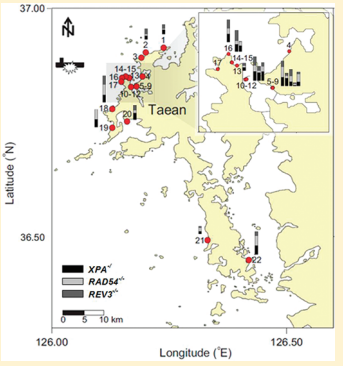
Supporting Information

ABSTRACT: The *Hebei Spirit* oil spill episode (December 7, 2007) has affected the western coastal area of South Korea; however, there is limited information on the potential toxicity of the oil spill to the ecosystem or humans. The potential toxicity of sediments collected from the affected area ($n = 22$) 2 years after the spill was evaluated. Acute lethal toxicity tests using *Vibrio fischeri* and *Moina macrocopa* and tests for genotoxicity and alteration of steroidogenesis using chicken DT40 cells and H295R cells, respectively, were conducted. Both crude and weathered oil extracts were evaluated in order to link the observed toxicity in the sediment extracts to the oil spill. Whereas toxicity to bacteria and daphnids was observed in only two elutriate samples, 10 of the 22 sediment extracts showed genotoxic potential in DT40 cells. The mechanisms of genotoxicity involved nucleotide excision repair ($XPA^{-/-}$), homologous recombination ($RAD54^{-/-}$), and translesion synthesis pathways ($REV3^{-/-}$). In addition, nine sediment extracts caused significantly greater production of E2 in H295R cells, and significant up-regulation of CYP19, CYP11B2, and 3βHSD2 by sediment extracts was observed. The pattern of toxicities observed in both crude and weathered oil samples was similar to that observed in the sediment extracts. The genotoxicity and endocrine-disruption potential of the sediment extracts suggest a need for long-term followup for such toxicity in humans and wildlife in this area.



INTRODUCTION

On December 7, 2007, the oil tanker M/V *Hebei Spirit*, which was carrying ~270000 ton of crude oil, collided with a crane barge near Taean on the west coast of South Korea (see Figure S1, Supporting Information). The collision resulted in punctures of three oil tanks and led to the spillage of approximately 10900 tons of crude oil, which spread out along a wide stretch of the coast.[1] Extensive cleanup activities were implemented immediately following the spill, and visual oiling and residues were removed from most of the beaches within a month of the episode.[2] However, major concerns regarding the long-term consequences of the spill remained, and a series of environmental impact assessments were subsequently conducted. Extensive monitoring has been implemented for oil content in seawater and pore water in the impacted areas and also in relatively undisturbed areas around the spill area.[1]

The *Hebei Spirit* oil spill episode is one of the largest oil spills that have occurred in Korea, with a volume approximately one-third that of the *Exxon Valdez* spill. Three different types of crude

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oils, namely, Iranian Heavy, UAE Upper Zakum, and Kuwait Export Crude, were spilled.[3] These types of crude oils contain various volatile organic compounds, such as benzene, toluene, ethylbenzene, and xylenes;[4] various polycyclic aromatic hydrocarbons (PAHs); and many other constituents. These compounds might have potentials for genotoxicity[5] and endocrine disruption.[6] Because sediments could provide binding sites for the hydrophobic fraction of the oil, sediment near the site of oil spillage could act as a reservoir for these compounds for a long time.[7] Constituents of spilled oil are reported to remain in the sediments of impacted sites for more than 5 years.[8]

Once spilled into the sea, oil is subjected to several chemical, physical, and biological processes, including evaporation, diffusion, (photo)oxidation, emulsification, microbial degradation, and sedimentation,[9] which is often called "weathering". The physicochemical properties of weathered oils significantly differ from those of fresh, unweathered oils.[10] Therefore, the toxicity of oils also will change during weathering. As a consequence of weathering, volatile, soluble, and low-molecular-weight fractions of oil dissipate. Weathered oil thus contains predominantly high-molecular-weight hydrocarbons, such as branched alkanes, (alkyl-substituted) cycloalkanes, and PAHs.[11]

Over the past 30 years, large-scale oil spills involving oil tanker accidents have occurred all over the world. Studies have been conducted to evaluate the influences of oil-related products on natural or in situ conditions.[12,13] Previous studies have documented the persistent nature of oil in the ecosystem and suggested potentials for long-term effects among marine wildlife even several years after a spill. For example, studies of the *Exxon Valdez* oil spill reported elevated cytochrome P4501A (CYP1A), aspartate aminotransferase, and lactate dehydrogenase enzyme activities in adult pigeon guillemots (*Cepphus Columba*) that were captured in the affected areas a decade after the spill.[14,15] Expression of CYP1A mRNA in harlequin ducks was greater in areas oiled by the *Exxon Valdez* spill relative to unoiled areas nearly 20 years after the spill.[16] Studies of the *Prestige* oil spill reported sublethal health impacts in adult gulls that bred in oiled colonies 17 months after the spill.[17,18]

In the present study, toxicities of sediments in and near the Taean area were determined 2 years after the oil spill. Toxicity was evaluated using luminescent bacteria and macroinvertebrates. In addition, chicken DT40 and H295R cell lines were employed to understand the genotoxic potentials and alteration of steroidogenesis of the sediments. The toxicities of a reference crude oil and artificially weathered oil were compared with those of environmental samples in order to associate the observed toxicity with the oil spill.

MATERIALS AND METHODS

Sediment Samples. Sediments were collected from 22 locations along the shore of the Taean area of the west coast of Korea, including the area adjacent to the location of the tanker collision (i.e., sites 1−20) and the west and east coasts of Anmyeon island (sites 21 and 22; see Figure S1, Supporting Information) in November 2009. Visual oiling was present in several locations, namely, Sinduri (sites 5−9) and Gaemokhang (sites 10−12). Because of the spill, it was reported that more than 150 km of coastline, including Kkotji (site 21) and Gomsum (site 22), was affected,[2] and the shoreline near Taean from Hagampo (site 2) to Padori (site 19) was severely affected.[1] All samples were collected using a core (10 cm in diameter and 30 cm in length), but only upper layer samples (top 10-cm layer) were used for evaluation in the present study. Samples were then freeze-dried using a cryo-desiccator (Bondiro, Ilsin Co., Busan, Korea) until further processing and analysis. Pore waters were obtained by centrifugation at 795*g* (i.e., 3000 rpm) immediately after sampling of the sediments.[19] Sediment elutriates were made by vigorously shaking sediments with 4 times the volume water for 1 h and separated[20] by centrifugation at 353*g* (i.e., 2000 rpm) for 30 min (for details, see the Supporting Information).

Crude and Weathered Oil Samples. Crude and weathered oil samples were used to investigate whether the toxicity from the sediments of the Taean area could be linked to the oil spill. Iranian heavy crude oil, one of the main oils that were spilled, was artificially weathered under controlled conditions in the laboratory to simulate evaporative weathering that might have occurred in crude oil in the Taean area. Approximately 50 mL of crude oil in a glass beaker was placed in a water bath at 70 °C. Weathering lasted until the weight loss of the crude oil was greater than 25%. Weathered oil used in this study had a 28.3% weight loss. The method used for artificial weathering in the present study might not closely simulate the natural weathering processes, but it might reflect the consequences of evaporation, which is an important component of oil weathering.[21] Average concentrations of major PAHs and alkylated PAHs in the both crude and weathered oil were measured and are reported in the Supporting Information, Figure S2.

Extract Preparation. Aliquots (20-g) of freeze-dried sediment samples were Soxhlet-extracted with 400 mL of high-purity dichloromethane (Burdick and Jackson, Muskegon, MI), and free sulfur was removed by treatment with acid-activated copper. Extracts were initially concentrated by rotary evaporator (N1000S, EYERA, Tokyo, Japan) and then by a gentle stream of nitrogen (N-evap, Organomation, Berlin, MA) to 1 mL. The final extract was prepared by replacing dichloromethane solvent with dimethyl sulfoxide (DMSO). Approximately 30 g of Iranian heavy crude oil and the artificially weathered oil (with 28.3% weight loss) were extracted with 300 mL of dichloromethane using the same method as described above. The extracts were concentrated by rotary evaporator. Again, dichloromethane solvent was replaced with DMSO, and the final extracts were concentrated to 1 mL by a gentle stream of nitrogen. All extracts of sediments or oil were used for cell line bioassays, and portions of the remaining sample extracts were further fractionated and analyzed for PAHs and alkylated PAHs, the analytical results of which are reported elsewhere.[22]

Microtox and *Moina macrocopa* Acute Toxicity Test. The toxicity of 12 pore water and 21 sediment elutriate samples was determined by the Microtox test employing *Vibrio fischeri*. The 81.9% Microtox Basic Test was conducted after 5 and 15 min of exposure at 15 ± 0.5 °C using the Microtox 500 Toxicity Analyzer (Azur Corp., Carlsbad, CA).

The 48-h acute toxicity test with *M. macrocopa* was conducted on elutriates in accordance with the procedure outlined by the U.S. Environmental Protection Agency[23] after minor modification. Briefly, four replicates with five neonates each (<24 h old) were exposed to five concentrations (6.25%, 12.5%, 25%, 50%, or 100%) and a control at 25 ± 1 °C. The number of immobile organisms was recorded 24 and 48 h after the exposure (for details, see the Supporting Information).

Chicken DT40 Mutant Cell Bioassay. Potencies of sediment extracts to induce genotoxic effects were determined with several

Table 1. Summary of Microtox Test, *M. macrocopa* Acute Test, Chicken DT40 Cell Bioassay, and H295R Cell Bioassay

	classification						
	Microtox test		*Moina macrocopa*	chicken DT40 cell bioassay	H295R cell bioassay		
sample	pore water[a]	elutriate[a]	acute test	genotoxicity[b]	E2 increase[c]	T decrease[d]	mRNA expression[e]
1	−	−	−	−	−	−	−
2	NA	−	−	−	−	−	−
3	NA	−	−	−	+	−	+
4	NA	−	−	−	+	−	+
5	−	−	−	++	−	−	−
6	−	−	−	+	−	−	−
7	−	−	−	−	−	−	−
8	NA	−	−	−	−	−	−
9	−	−	−	−	−	−	−
10	NA	NA	−	++	−	−	−
11	+	−	−	+	−	−	−
12	−	−	−	+	−	−	−
13	−	−	−	−	−	−	−
14	−	−	−	+	+	+	+
15	−	−	−	−	+	−	+
16	NA	−	−	++	+	+	+
17	NA	−	−	−	+	+	+
18	NA	−	−	++	+	−	+
19	NA	−	+	+	−	−	−
20	−	−	−	−	+	−	+
21	NA	−	−	−	−	−	−
22	−	−	−	++	+	−	+

[a] Toxicity was observed, +; toxicity was not observed, −; not available, NA. [b] Chicken DT40 mutant cell bioassay genotoxicity classification according to hypersensitivity exhibition of DT40 cells that were deficient in $XPA^{-/}$, $RAD54^{-/-}$, or $REV3^{-/-}$ to 8 mg of sediment (dry weight)/mL: no hypersensitivity, −; one-mutant hypersensitivity, +; two-mutant hypersensitivity, ++; three-mutant hypersensitivity, +++. [c] H295R cell bioassay E2 production classification according to statistically significant increase relative to solvent control of H295R cells after exposure to 4 mg of sediment (dry weight)/mL: no change, −; significant increase, +. [d] H295R cell bioassay T production classification according to statistically significant decrease relative to solvent control of H295R cells after exposure to 4 mg of sediment (dry weight)/mL: no change, −; significant decrease, +. [e] H295R cell bioassay mRNA classification according to statistically significant change relative to solvent control of H295R cells after exposure to 4 mg sediment (dry weight)/mL: no change, −; significant change in mRNA, +.

DNA-repair-deficient clones of chicken DT40 B-lymphocyte cell lines. First, 'noncytotoxic' doses of sediment extract were determined, where less than 20% decrease of cell viability was observed in wild-type cells. When the cell viability of a given mutant was reduced by more than a factor of 3 compared to that of the wild type after exposure to the noncytotoxic dose, the mutant was defined as hypersensitive, and the sediment sample was classified as genotoxic. Based on this approach, genotoxic sediments were detected by simply monitoring the differences in cellular proliferation rates between the wild-type cells and the mutants. Cell viability was indirectly measured by the amount of adenosine-5′-triphosphate (ATP) in cell lysates as previously described.[24] Details of this procedure can be found in the Supporting Information. We conducted this assay two times with duplicate exposure ($n = 4$).

H295R Cell Bioassay. The effects on the hormone production and expression of mRNAs involved in steroidogenesis were determined with H295R cell bioassays. H295R (human adrenocortical carcinoma cell line) were obtained from the American Type Culture Collection (Manassas, VA) and cultured by methods described elsewhere.[25] To minimize the influence of cytotoxicity on H295R cells, viability was checked with an MTT bioassay,[26] and noncytotoxic doses (>80% of survival) were determined for evaluation of effects on hormone production[27] and steroidogenic gene transcription.[25] In brief, H295R cells were exposed to sediment extracts [4 mg of sediment (dry weight)/mL] for 48 h, and culture medium and remaining cells were used for hormone measurements and gene transcription analysis, respectively. Hormones were measured by competitive enzyme-linked immunosorbent assay (ELISA), and expression of mRNA was quantified by real-time polymerase chain reaction (PCR). (For details, see the Supporting Information.)

Statistical Analyses. Microtox test results were analyzed using Microtox Omni Software for Windows, version 1.16 (Azur Corp.), and the median inhibitory concentrations (IC_{50} values, the concentrations producing a 50% reduction in light) were calculated. The median effective concentrations (EC_{50}) and confidence intervals (CIs) of *M. macrocopa* acute toxicity tests were calculated using ToxStat (version 3.5, West, Cheyenne, WY). Differences in responses among different cell lines were evaluated by one-way ANOVA followed by Dunnet's test using SPSS 15.0 for Windows (SPSS, Chicago, IL). P values less than 0.05 were considered to be statistically significant.

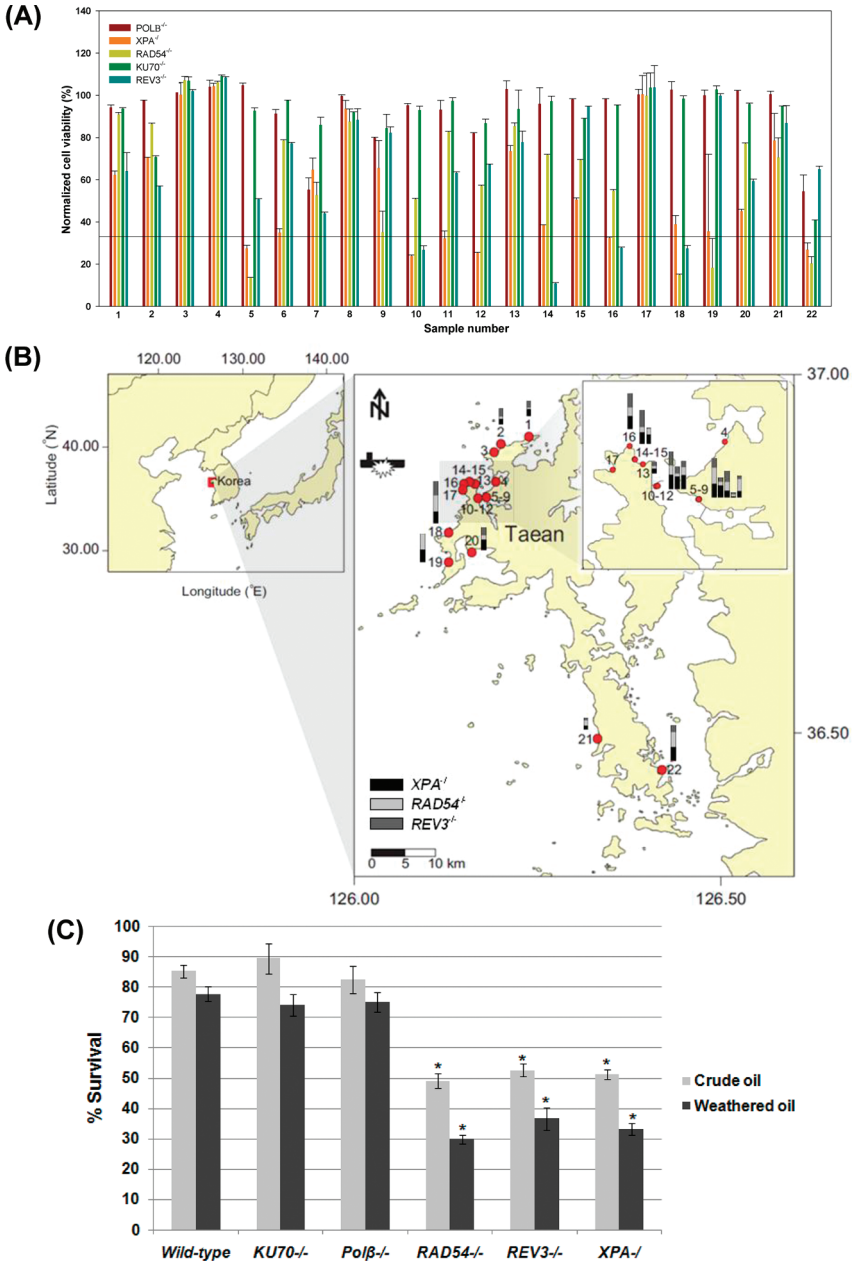

Figure 1. Genotoxic effects of sediment extracts. (A) Normalized cell viability of five mutant chicken DT40 cells (KU70$^{-/-}$, POL$\beta^{-/-}$, RAD54$^{-/-}$, REV3$^{-/-}$, and XPA$^{-/}$) relative to wild type in response to exposure to 8 mg of sediment (dry weight)/mL. Lower lines indicate hypersensitivity. (B) Distributions of genotoxic samples based on the responses of three hypersensitive chicken DT40 cells (XPA$^{-/}$, RAD54$^{-/-}$ and REV3$^{-/-}$). Bars indicate the mortality of each mutant; therefore, longer bars mean greater genotoxic potential. (C) Cell viability of wild-type and five DT40 mutant cells (KU70$^{-/-}$, POL$\beta^{-/-}$, RAD54$^{-/-}$, REV3$^{-/-}$, and XPA$^{-/}$) after exposure to 30 mg/mL crude and weathered oil. Data are expressed as means ± standard deviations of duplicate exposures that were repeated two times. *$p < 0.05$, compared to that of wild-type cells by one-way ANOVA.

RESULTS

Microtox and *Moina macrocopa* Acute Toxicity Test. IC_{50} values could not be derived for pore water or elutriates in the Microtox test, except one pore for water (Table 1). The pore water of Gaemokhang sediment (site 11) showed a 5-min IC_{50} (95% CI) of 53.5% (29.4−97.3%) and a 15-min IC_{50} (95% CI) of 38.4% (19.7−74.8%).

Acute toxicity to *M. macrocopa* was observed in elutriates collected from only one location, Padori (site 19) (Table 1). The 48-h EC_{50} (95% CI) values for the elutriate samples from the middle and deeper layers of sediments from Padori were 19.8% (4.55−85.8%) and 46.1% (10.7−198%), respectively.

Chicken DT40 Mutant Cell Bioassay. The cytotoxicity of the wild-type cells treated with sediment extracts was evaluated (Figure S3 in the Supporting Information), and only the non-cytotoxic dose of sediment extracts [i.e., 8 mg of sediment (dry weight)/mL] was used to determine genotoxic potential. The cell viability analysis using five mutant cells [KU70$^{-/-}$, DNA polymerase β (POL$\beta^{-/-}$), RAD54$^{-/-}$, REV3$^{-/-}$, and Xeroderma pigmentosum complementation group A$^{-/}$ (XPA$^{-/}$)] in comparison with wild type indicated that 10 of the 22 organic

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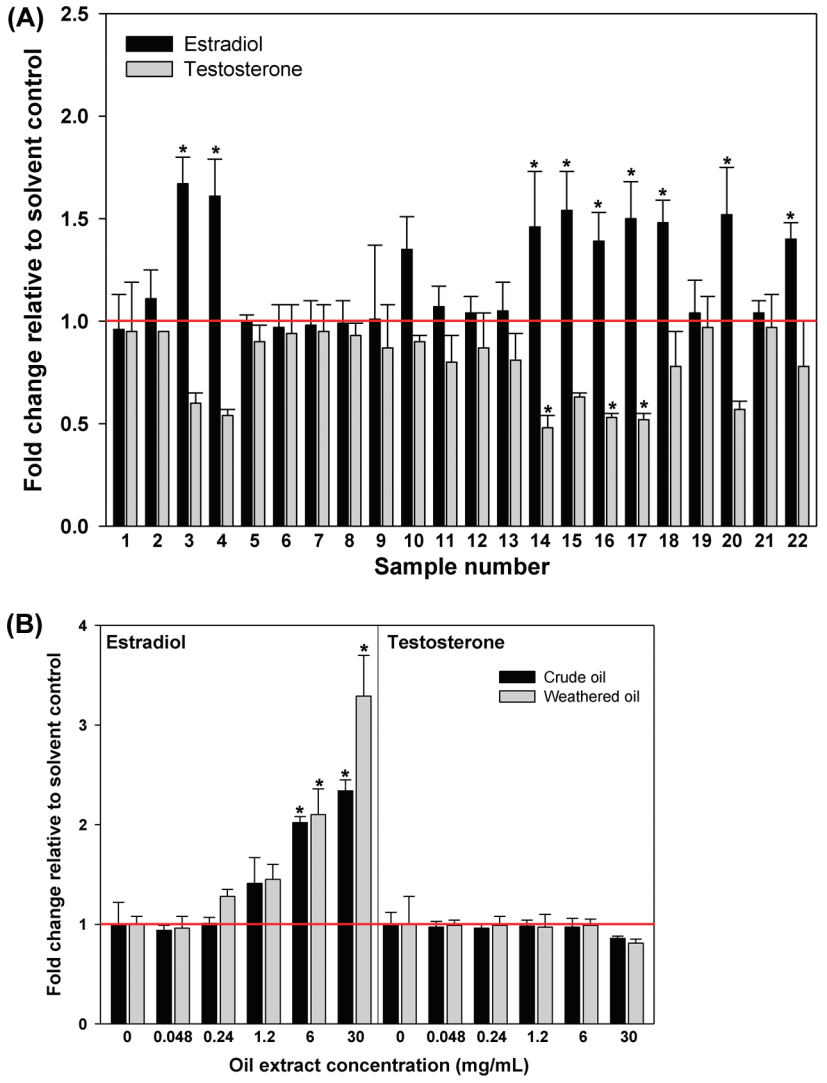

Figure 2. Effects of sediment, crude oil, and weathered oil extracts on hormone production in H295R cells. Concentrations of 17β-estradiol and testosterone in H295R cells after exposure to (A) 4 mg of sediment (dry weight)/mL (upper layer; $n = 22$) and (B) crude and weathered oil. Data are expressed as means ± standard deviations of three replicate exposures (data are expressed as fold induction relative to appropriate solvent control; DMSO fold induction = 1). *$p < 0.05$, compared with DMSO control by one-way ANOVA.

extracts of sediments exhibited genotoxicity (Figure 1A). The DT40 cells that were deficient in nucleotide excision repair ($XPA^{-/}$), homologous recombination ($RAD54^{-/-}$), and translesion synthesis ($REV3^{-/-}$) pathways exhibited a hypersensitivity to most of the sediment extracts (Figure 1A). Those locations where sediments exhibited genotoxicity in the susceptible chicken DT40 mutant cells such as $XPA^{-/}$, $RAD54^{-/-}$, or $REV3^{-/-}$ are given in Figure 1B. Among the sediment extracts, those collected from Sinduri (site 5), Gaemokhang (site 10), Ouetaebae (site 16), Mohanghang (site 18), and Gomsum (site 22) exhibited the greatest genotoxicity (Figure 1B and Table 1). The extracts of the crude and weathered oils exhibited similar patterns of hypersensitivity. Extracts of both crude and weathered oils caused hypersensitivity in DT40 cells that were deficient in the XPA, RAD54, and REV3 pathways (Figure 1C).

H295R Cell Bioassay. Viability of cells treated with sediment extracts was evaluated by use of the MTT assay (Figure S4 in the Supporting Information), and only the noncytotoxic concentrations of sediment extracts that resulted in >80% survival [4 mg of sediment (dry weight)/mL] were used for further evaluations. Significant effects on hormone production were observed after exposure to most of the sediment extracts (Figure 2A). Greater production of 17β-estradiol (E2) was observed for extracts of Guryepo (site 3), Padori (site 4), Naetaebae (sites 14 and 15), Ouetaebae (site 16), Gurumpo (site 17), Mohanghang (site 18), Sinduk salt field (site 20), and Gomsum (site 22) sediments (Figure 2A and Table 1). Significantly less production of testosterone (T) was observed in Naetaebae (site 14), Ouetaebae (site 16), and Gurumpo (site 17) sediment extracts (Figure 2A and Table 1). The locations where sediment extracts caused lower production of testosterone (T) coincided with those of greatest E2 production. After exposure to extracts of both crude and weathered oil samples, E2 production significantly increased at 6 mg of oil extracts/mL and greater. The effect on greater E2 production was more pronounced with the weathered oil extract compared to the crude oil extract (Figure 2B). Although not statistically

significant, production of T was inversely proportional to concentration of oil extract (Figure 2B).

The mRNA expression of six major steroidogenic genes was also measured in response to the organic contaminants present in the sediment extracts. Of the 22 sediment extracts evaluated, 9 samples significantly altered transcription of at least one gene relative to that of the solvent control. Significant increases in the expression of CYP19, CYP11B2, and 3βHSD2 mRNA were frequently observed after exposure to sediment extracts. Similarly, extracts of crude and weathered oil samples also showed enhanced expression of all three of these mRNAs (Table S2, Supporting Information).

DISCUSSION

Potential adverse effects remain in and around the Taean area 2 years after the *Hebei Spirit* oil spill. The sediments are not currently acutely toxic to the bacterium and invertebrates. Only two samples showed changes in the production of light by the bacterium *V. fischeri* or immobilization of *M. macrocopa* after exposure to pore water or elutriates (Table 1). These results are consistent with those of other studies, which reported that acute lethal toxicity of pore water to *V. fischeri* and aquatic organism decreases as weathering proceeds.[21,28] Crude oil contains petroleum hydrocarbons that can be dissipated, but are acutely toxic. Because of removal of such compounds during weathering, the acute toxicity is reduced significantly, rather soon after the spill has occurred. The fact the limited acute toxicity was observed in the microorganisms and daphnids in most of the samples indicates that weathering might have taken place.

Some constituents of oil can persist longer in the environment and cause chronic adverse effects. An oil spill in water can lead to increases in concentrations of PAHs and alkylated PAHs in water, which are reported to persist in sediments for longer than 2 years.[29] Persistence of these compounds can result in several negative impacts on organisms,[30] because of their carcinogenic, mutagenic, or endocrine-disruption potentials.[5,6] To assess these possibilities, we employed the chicken DT40 mutant cell bioassay for genotoxicity and H295R cell bioassay for endocrine disruption. The chicken DT40 mutant cell bioassay is a sensitive and fast screening method while also being capable of characterizing genotoxicity. This novel bioassay has been applied to environmental contaminants such as sodium arsenite[24] and polybrominated diphenyl ethers[31] for screening and characterizing genotoxicity. This study is the first application of the novel chicken DT40 mutant cell bioassay that evaluates genotoxic potentials of contaminated environmental samples (sediment extracts).

In most samples that showed genotoxic potential (Figure 1A, B), the DT40 cells deficient in nucleotide excision repair ($XPA^{-/}$), homologous recombination ($RAD54^{-/-}$), or translesion synthesis ($REV3^{-/-}$) exhibited hypersensitivity, suggesting underlying genotoxicity mechanisms. Oil includes genotoxic constituents, such as PAHs, alkylated PAHs, and metals. The oil spilled from the *Hebei Spirit* was reported to also contain these compounds.[32,33] Exposure to benzo[*a*]pyrene and benz[*a*]anthracene induces double-strand breaks in CHO cells.[34,35] PAH metabolites also have genotoxic potentials. Formation of covalent adducts between DNA and 1-hydroxypyrene has been reported.[36] Greater genotoxic potency was also reported in methyl and benzo derivatives of chrysene when compared to that of parent compound, suggesting greater genotoxic potentials of alkylated PAHs.[37] For both crude and weathered oils, the DT40 cells deficient in XPA, RAD54, and REV3 pathways exhibited hypersensitivity (Figure 1C), which was the same pattern as was observed from the environmental samples. This observation suggests that genotoxicity of sediments of the Taean area might be due to the oil spill that took place 2 years ago.

The fact that significantly greater E2 and significantly lower T production was observed in H295R cells after exposure to the sediment extracts indicates endocrine-disrupting potential of the oil spill (Figure 2A). PAHs could result in reproduction-related effects[38] and alteration of steroidogenesis.[39] The fact that the exposure to both crude and weathered oil extracts resulted in greater E2 production by H295R cells (Figure 2B) suggests that the observed endocrine-disrupting effects from the sediment extract samples in and near Taean area might be linked to the oil spill.

Significant upregulation of three major steroidogenic mRNAs, namely, CYP19, CYP11B2, and 3βHSD2, by both sediment and oil extracts supports the endocrine-disruption potentials of several sediment extracts. CYP19 mRNA regulates the translation to the aromatase enzyme, which is responsible for converting androstenedione into estrone and T into E2. In the present study, five sample extracts showed significant increases in CYP19 mRNA expression and E2 production, which was accompanied by a decrease of T. Significant increases in 3βHSD2 and CYP11B2 might also influence the balance of steroid hormones. 3βHSD2 catalyzes the conversion of dehydroepiandrostenone to androstenedione. CYP11B catalyzes the conversion of 11-deoxycorticosterone to aldosterone through the intermediates corticosterone and 18-hydroxycorticosterone. Bláha et al.[40] reported an increase of CYP11B2 mRNA expression in H295R cells that were exposed to extracts of freshwater pond sediments. They associated this observation with the presence of relatively higher concentrations [18 μg of total PAHs/g of sediment (dry weight)] of PAHs and persistent chlorinated chemicals (e.g., polychlorinated biphenyls and organochlorine pesticides) in the pond sediments.

A previous investigation in the Taean area documented that 16 PAHs and alkylated PAHs were detected at greater than 20 ng/g (dry weight) in the majority of sampling area in June 2009.[32] This result supports the link between the oil spill and the observed toxicity in the present study. The toxicity of the Kkotji (site 21) sample, however, could not be explained by PAHs because the concentrations of PAHs were quite low in that sample.[32] Other types of contaminants that might be introduced by human activities cannot be ignored as a cause of the observed toxicity at Kkotji.

The observation of the genotoxic potential and alteration of steroidogenesis in sediment extracts suggests that the impact of an oil spill could last for at least 2 years even after extensive cleanup activities, although the influence of other anthropogenic input cannot be completely ruled out. Long-term followup studies on genotoxic impacts and alteration of steroidogenesis on humans and wildlife should be implemented.

ASSOCIATED CONTENT

Supporting Information. Supporting Materials and Methods and Supporting Results sections, with corresponding reference list. This material is available free of charge via the Internet at http://pubs.acs.org.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper published August 5, 2011 with an error in Table 1, reference 19 appearing as a duplicate of reference 2, and an incomplete reference 22. The correct version published August 10, 2011.

SUPPORTING INFORMATION

Genotoxicity and endocrine disruption potentials of sediment near an oil spill site: 2 years after the *Hebei Spirit* oil spill

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Contents of Supporting Information

Supporting Materials and Methods

Sediment samples

Sediments were collected from 22 locations along the shore of the Taean area of the west coast of Korea which included the area adjacent to the location of the tanker collision (e.g., St. 1 – 20) and the west and east coast of Anmyeon island (St. 21 – 22).

Sediment elutriates were prepared by combining in a sediment-to-water ratio of 1:4 on a volume basis at room temperature (*1*). Briefly, the mixture was stirred vigorously for 30 min with shaking incubator (Lab Companion^{TM} SI-600R, Daejeon, Korea). After 10 min of pause, the mixture was again vigorously shaken for additional 30 min to ensure complete mixing. Supernatant was separated after 1 h settling, and was used as sediment elutriates.

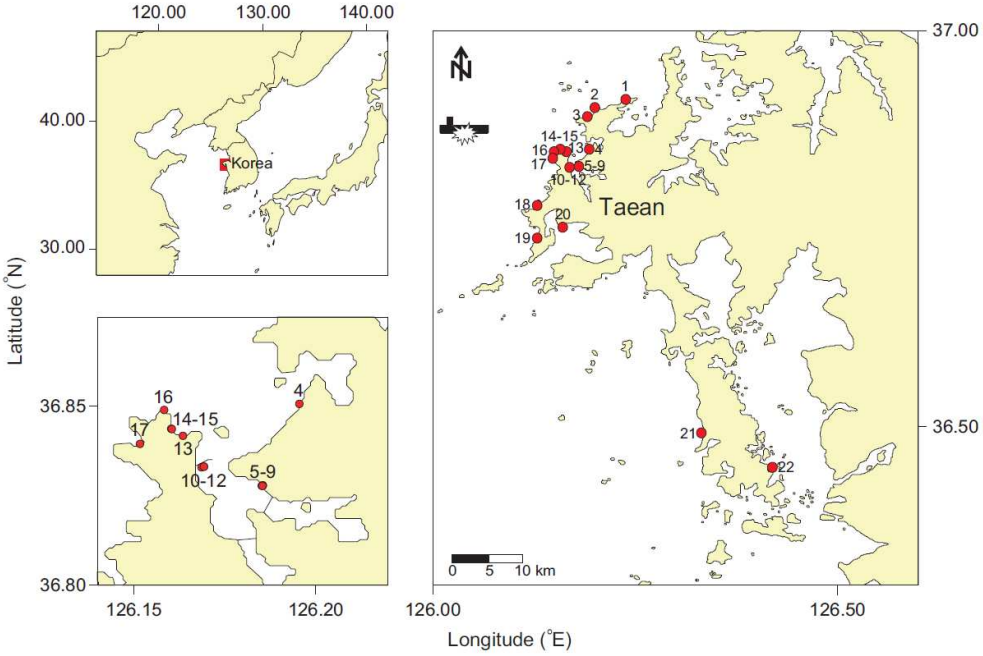

Figure S1. Sediment sampling locations (n=22) in the Taean area, Korea.

Crude and weathered oil samples

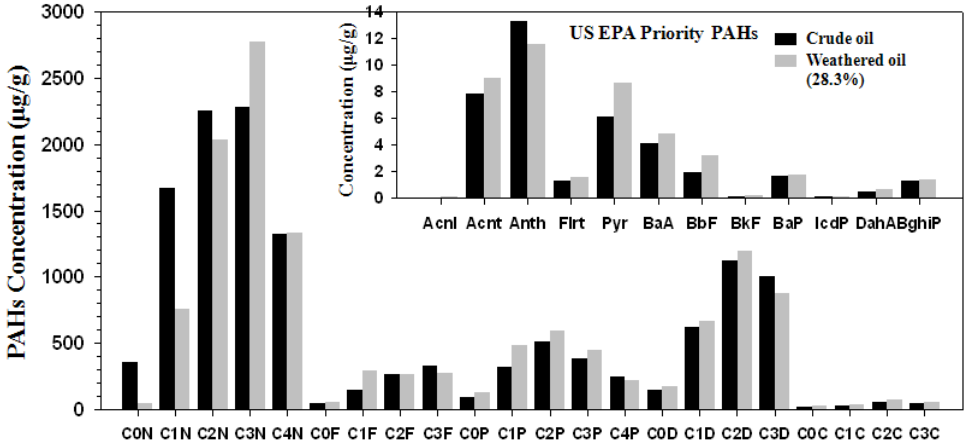

Figure S2. Average concentrations (µg/g) of 16 PAHs and alkylated PAHs in Iranian Heavy crude oil and weathered oil.

Abbreviation: Acnl-Acenaphthylene, Acnt-Acenaphthene, Anth-Anthracene, Flrt-Fluoranthene, Pyr-Pyrene, BaA-Benz[*a*]anthracene, BbF-Benzo[*b*] fluoranthene, BkF-Benzo[*k*]fluoranthene, BaP-Benzo[*a*]pyrene, IcdP-Indeno [1,2,3-cd]pyrene, DahA-Dibenzo[a,h]anthracene, BghiP-Benzo[ghi]perylene, N-Naphthalene, F-Fluorene, P-Phenanthrene, D-Dibenzothiophene, C-Chrysene

Microtox® and Moina macrocopa acute toxicity test

Four replicates with five neonates each (<24 h old) were exposed to five concentrations (6.25%, 12.5%, 25%, 50%, or 100% elutriates) and a control at 25±1 ^{o}C. The number of immobile organisms was recorded at 24 and 48 h after the exposure. Test organisms were not fed and test solutions were not renewed during this period. Water quality parameters, including pH, temperature, conductivity, and dissolved oxygen were measured before and 48 h after the exposure. To assure comparable sensitivity of test organisms over time, monthly reference tests using zinc chloride used as a reference toxicant were conducted (data not shown).

Chicken DT40 mutant cell bioassay

In brief, five isogenic DT40 mutant cells were used, each of which is defective of one of the major DNA damage pathways, including base excision repair (*2*), nucleotide excision repair (*3*), homologous recombination (*4*), non-homologous end-joining (*5*), and translesion DNA synthesis (*6*) (Table S1). Approximately 10^3 cells were seeded into 24-well plates containing 1 ml of culture medium per well, and were exposed to five concentrations of sediment extracts for 72 h. ATP assays were carried out in 96-well plates using a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega Corp., Madison, WI, USA) with a plate reader (Infinite® 200, Tecan, Männedorf, Switzerland).

Table S1. DNA repair genes mutated in DT40 clones.

Gene	Function	Reference
KU70	Non-homologous end-joining, which repairs double-strand breaks at any cell-cycle phase	(*5*)
POLβ	Base excision repair, which repairs single-strand breaks or base damage	(*2*)
RAD54	Homologous recombination	(*4*)
REV3	Translesion DNA synthesis	(*6*)
XPA	Nucleotide excision repair, which eliminates bulky base damage	(*3*)

Hormone measurement of H295R cell

The solvent concentration used for preparation of the sediment extract solution was limited to <0.1% v/v. 17β-estradiol (E2) and testosterone (T) were measured following methods described by Hecker et al. (*7*). H295R cells were seeded into 24-well plates and exposed for 48 h to five concentrations of sediment extracts, plus a negative control (procedural blank) or a solvent control (0.1% DMSO). After 48 h, the culture medium was collected and kept frozen at -80 ^{o}C until media extraction. Frozen medium was thawed on ice, and 500 µl medium was extracted twice with 2.5 ml diethyl ether each. The solvent phase containing target hormones was evaporated under a gentle stream of nitrogen, and the residue was reconstituted in 300 µl Enzyme-linked immunosorbent assay (ELISA) buffer (Cayman Chemical, Ann Arbor, MI, USA) and frozen at -80 ^{o}C until further analysis. Medium extracts were diluted 1:75 and 1:1 for T and E2, respectively. Hormones were measured by competitive ELISA following the manufacturer's recommendations (Cayman Chemical; Testosterone [Cat # 582701], 17β-Estradiol [Cat # 582251]).

Quantitative PCR assay of H295R cell

Transcription of six steroidogenic genes (*CYP17* (Assay ID: Hs01124136_m1), *CYP19* (Assay ID: Hs00903413_m1), *CYP11A1* (Assay ID: Hs00167984_m1), *CYP11B2* (Assay ID: Hs01597732_m1), *3βHSD2* (Assay ID: Hs00605123_m1), *17βHSD4* (Assay ID: Hs00264973_m1) plus one housekeeping gene (*β-actin* (Assay ID: Hs99999903_m1)) were quantified in H295R cells exposed to sediment extracts (4 mg sediment d.w./mL) following the method outlined by Hilscherova et al. (*8*). Briefly, cell suspension (3×10^5 cells/ml) was transferred to each well of a 24-well plate. After 24 h, cells were exposed to sediment extracts for another 48 h, and then total RNA was isolated from each well using a total RNA isolation kit (Agilent Technologies, Ontario, CA, USA). Two micrograms of cellular total RNA for each sample was used for reverse transcription using the iScript^{TM} cDNA Synthesis Kit (BioRad, Hercules, CA, USA). The ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time PCR. PCR reaction mixtures contained 10 µl TaqMan Gene Expression Master Mix, 1 µl TaqMan gene expression assay, 5 µl RNase-free water, and 4 µl complementary DNA template. The thermal cycle profile was: 50 ^{o}C for 2 min, 95 ^{o}C for 10 min, 40 cycles at 95 ^{o}C for 15 s, and 60 ^{o}C for 1 min.

Expression of mRNA was quantified by use of the threshold cycle, *C*t method. *C*t values for each gene of interest were normalized to that of *β-actin*. Normalized values were then used to calculate the degree of induction or inhibition and expressed as a "fold difference" compared to normalized control values. Therefore, all data were statistically analyzed as "fold induction" between the exposed and control cultures. Expression of mRNA was measured in triplicate for each treatment and control.

Supporting Results

Chicken DT40 mutant cell bioassay

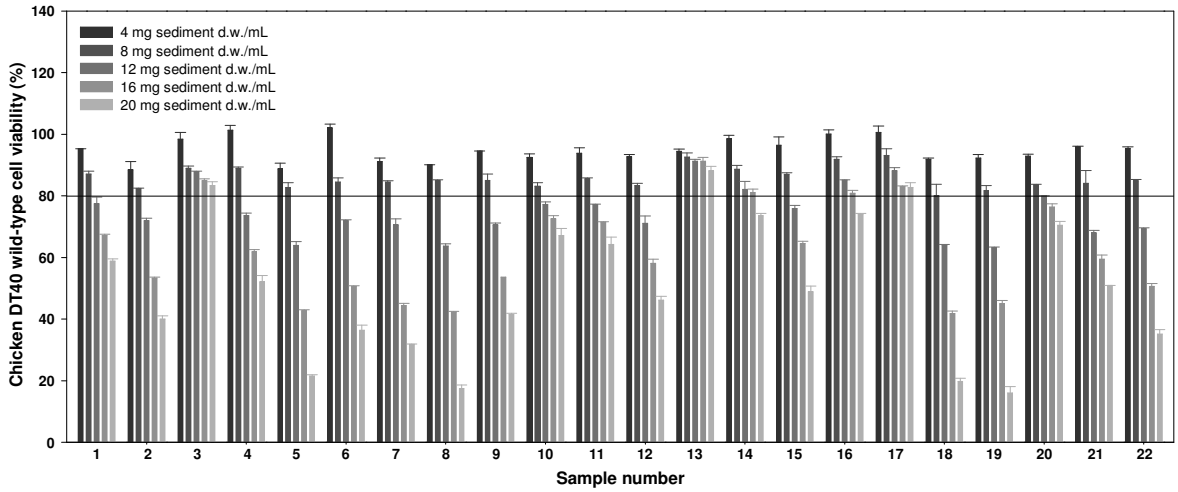

Figure S3. The viability of the wild-type chicken DT40 cells after exposure to sediment extracts (upper layer; n=22). The cell viability of wild-type cells treated with sediment extracts (8 mg sediment d.w./mL) showed more than 80% survival, then this non-cytotoxic doses were used to determine genotoxic potential.

MTT assay of H295R cell

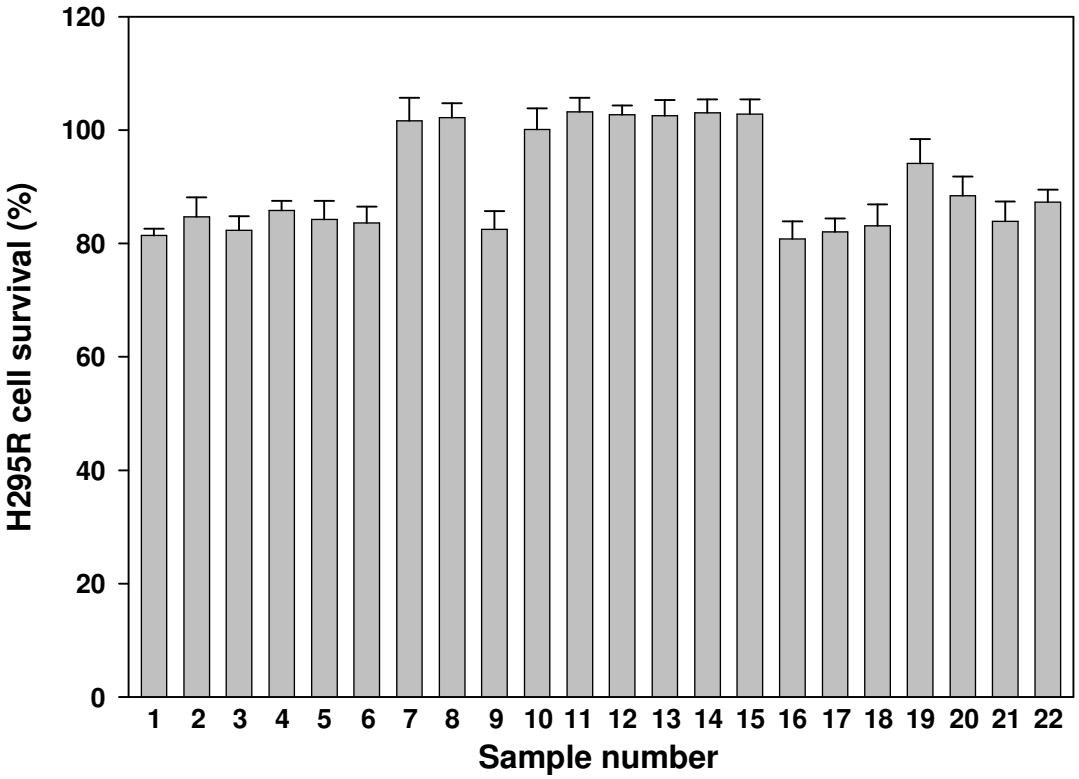

Figure S4. The viability of the H295R cells after exposure to sediment extracts (upper layer; n=22). The cell viability of wild-type cells treated with sediment extracts (4 mg sediment d.w./mL) showed more than 80% survival, then this non-cytotoxic doses were used to determine genotoxic potential.

Quantitative PCR assay of H295R cell

Table S2. Expression of CYP17, CYP19, CYP11A1, CYP11B2, 3βHSD2, and 17βHSD4 mRNAs in steroidogenic pathways of H295R cells which were exposed to 22 sediment samples collected from Taean area, Korea

Sample	mRNA					
	CYP17	*CYP19*	*CYP11A1*	*CYP11B2*	*3βHSD2*	*17βHSD4*
1	0.78 ± 0.24	1.41 ± 0.05	1.22 ± 0.05	0.95 ± 0.25	1.26 ± 0.30	0.65 ± 0.16
2	1.22 ± 0.40	1.59 ± 0.28	0.93 ± 0.19	1.18 ± 0.54	0.96 ± 0.12	0.81 ± 0.26
3	0.73 ± 0.21	1.79 ± 0.37	0.98 ± 0.49	1.84 ± 0.08 *	1.22 ± 0.55	1.50 ± 0.69
4	0.92 ± 0.38	1.84 ± 0.63	1.08 ± 0.34	1.86 ± 0.37 *	1.07 ± 0.52	0.68 ± 0.32
5	0.90 ± 0.75	1.05 ± 0.40	0.97 ± 0.53	1.08 ± 0.69	0.85 ± 0.22	0.46 ± 0.28
6	0.96 ± 0.30	1.28 ± 0.21	0.64 ± 0.12	1.18 ± 0.17	0.62 ± 0.15	0.59 ± 0.26
7	0.99 ± 0.12	0.86 ± 0.40	0.72 ± 0.37	0.99 ± 0.16	0.93 ± 0.28	0.61 ± 0.13
8	0.92 ± 0.17	0.75 ± 0.22	0.65 ± 0.18	1.16 ± 0.53	0.80 ± 0.25	0.49 ± 0.14
9	0.88 ± 0.55	1.01 ± 0.34	1.03 ± 0.21	0.89 ± 0.20	1.21 ± 0.36	0.75 ± 0.25
10	0.88 ± 0.15	0.81 ± 0.17	0.87 ± 0.47	0.95 ± 0.34	0.84 ± 0.28	0.67 ± 0.35
11	1.01 ± 0.20	1.10 ± 0.47	0.95 ± 0.55	0.94 ± 0.31	0.75 ± 0.10	0.50 ± 0.31
12	1.36 ± 0.34	0.99 ± 0.33	1.22 ± 0.49	0.67 ± 0.35	1.07 ± 0.34	0.78 ± 0.43
13	1.17 ± 0.54	1.43 ± 0.12	1.05 ± 0.33	1.19 ± 0.10	1.10 ± 0.48	0.53 ± 0.29
14	1.44 ± 0.41	1.94 ± 0.05 *	1.14 ± 0.25	0.82 ± 0.19	0.81 ± 0.02	0.63 ± 0.28
15	1.31 ± 1.10	1.95 ± 0.45 *	1.05 ± 0.70	1.52 ± 0.54	1.24 ± 0.25	0.95 ± 0.81
16	0.52 ± 0.14	2.38 ± 0.45 *	1.26 ± 0.34	1.98 ± 0.40 *	0.62 ± 0.15	0.76 ± 0.26
17	0.93 ± 0.31	2.31 ± 0.16 *	1.36 ± 0.40	1.90 ± 0.46 *	0.54 ± 0.13	0.96 ± 0.29
18	0.73 ± 0.06	1.92 ± 1.06	1.37 ± 0.34	1.90 ± 0.58 *	0.69 ± 0.24	0.80 ± 0.34
19	0.54 ± 0.10	1.57 ± 0.40	0.95 ± 0.25	1.39 ± 0.39	0.82 ± 0.29	0.83 ± 0.04
20	1.22 ± 0.77	1.97 ± 0.45 *	1.21 ± 0.27	1.67 ± 0.18	2.75 ± 0.21 *	0.72 ± 0.41
21	0.56 ± 0.09	1.08 ± 0.04	1.36 ± 0.47	1.36 ± 0.18	0.76 ± 0.36	0.72 ± 0.17
22	0.70 ± 0.02	1.87 ± 0.30	1.11 ± 0.45	0.90 ± 0.25	2.63 ± 0.91 *	0.94 ± 0.39
Crude	0.39 ± 0.01	2.41 ± 0.62 *	0.86 ± 0.07	2.24 ± 0.46 *	2.00 ± 0.10 *	0.47 ± 0.13
Weathered	0.37 ± 0.23	3.21 ± 0.54 *	1.02 ± 0.23	2.92 ± 0.25 *	2.84 ± 0.13 *	0.54 ± 0.30

All mRNA expression values are fold changes relative to DMSO the solvent control (=1), and are given as means and SDs.

* indicates statistically significant differences at $p < 0.05$.

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